P2Y2 purinergic receptor modulates virus yield, calcium homeostasis, and cell motility in human cytomegalovirus-infected cells

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Human cytomegalovirus (HCMV) manipulates many aspects of host cell biology to create an intracellular milieu optimally supportive of its replication and spread. Our study reveals that levels of several components of the purinergic signaling system, including the P2Y2 and P2X5 receptors, are elevated in HCMV-infected fibroblasts. Knockdown and drug treatment experiments demonstrated that P2Y2 enhances the yield of virus, whereas P2X5 reduces HCMV production. The HCMV IE1 protein induces P2Y2 expression; and P2Y2-mediated signaling is important for efficient HCMV gene expression, DNA synthesis, and the production of infectious HCMV progeny. P2Y2 cooperates with the viral UL37x1 protein to regulate cystolic Ca^{2+} levels. P2Y2 also regulates PI3K/Akt signaling and infected cell motility. Thus, P2Y2 functions at multiple points within the viral replication cycle to support the efficient production of HCMV progeny, and it may facilitate in vivo viral spread through its role in cell migration.

cytomegalovirus | purinergic receptors | calcium homeostasis | cell migration

Human cytomegalovirus (HCMV) is a β-herpesvirus that infects a large percentage of the adult population worldwide. Infection in immunocompetent people is typically asymptomatic. In contrast, HCMV is a leading opportunistic pathogen in immunosuppressed individuals (1–3), and a major infectious cause of birth defects (4).

HCMV perturbs cellular homeostasis to support optimal viral replication and spread. One mechanism utilized by the virus to change the biology of infected cells is via the regulation of expression levels and activities of cell-surface proteins (5, 6). This report investigates the role of cell-surface purinergic receptors during HCMV infection. Purinergic receptors are activated by extracellular ATP/ADP (P2 receptors) or adenosine (P1 receptors). P2 purinergic receptors are further divided into ionotropic P2X and metabotropic P2Y families. P2X receptors are ATP-gated ion channels and P2Y receptors are G protein-coupled receptors that are activated by adenine and uridine nucleotides or nucleotide sugars. Seven subtypes of P2X receptors (P2X1 to -7) and 8 P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 to -14) have been identified, many of which are therapeutic targets (7, 8). P2Y2 and P2X5 are the focus of this report.

Signaling via P2Y receptors triggers the activation of a heterotrimeric G protein, which in turn activates phospholipase C (PLC), leading to the generation of diacylglycerol and inositol 1,4,5-triphophosphate (IP_3) (9). Diacylglycerol stimulates protein kinase C (PKC) (10), which is known for its role in DNA synthesis, proliferation, and migration of fibroblasts and other cells (11). IP_3 mobilizes cytosolic calcium from the smooth endoplasmic reticulum. In lung fibroblasts P2Y2 is the only purinergic receptor subtype that, when activated, causes the mobilization of cytosolic Ca^{2+} (12). P2Y2-mediated intracellular Ca^{2+} increases have been implicated in the proliferation and migration of hepatocellular carcinoma cells in mice (13), and in the migration of ovarian carcinoma cells (14). Moreover, P2Y2 signaling was found to stimulate HIV-1 viral fusion through the activation of proline-rich tyrosine kinase 2 (15).

P2X receptors differ from their P2Y counterparts in ligand selectivity. While P2Y receptors recognize a wide range of agonists, P2X receptors are activated only by ATP (16). These receptors are assembled as trimeric proteins, and P2X5 mainly functions as a heterotrimer that can include P2X1, P2X2, or P2X4 subunits (17). The expression of P2X5 receptors is normally restricted to the trigeminal mesencephalic nucleus of the brainstem, sensory neurons, cervical spinal cord, and some blood vessels (18). Activation of the P2X5 receptor causes an influx of cations (Na^+, K^+, and Ca^{2+}) across the plasma membrane (19), and P2X5 expression in atypical locations has been linked to cancer (20, 21). The tissue specificity of P2X5 may provide at least a partial explanation for the divergent effects of the hepatatis C virus (HCV) and human papillomavirus (HPV) on the P2X5 expression seen in infected cells (22, 23).

Purinergic receptors have been reported to modulate bacterial (24, 25), protozoal (26, 27), and viral (15, 28–30) infections. Knowing that purinergic receptors can regulate cellular Ca^{2+} levels and that an increase in cytosolic Ca^{2+} has been observed following HCMV infection (31), one might speculate that purinergic receptors could influence HCMV infection. It has been reported previously that the expression of several members of the purinergic receptor family is elevated in HCMV-infected cells (30, 32), but their role during infection has not been determined. The

Significance

Human cytomegalovirus (HCMV) infection is ubiquitous and can cause life-threatening disease in immunocompromised patients, debilitating birth defects in newborns, and has been increasingly associated with chronic conditions, such as atherosclerosis and cancer. Such broad clinical consequence result in part from the modulation of multiple host cell processes. This study shows that functions of cellular purinergic receptors are usurped in HCMV-infected cells to promote efficient production of HCMV progeny and possibly to facilitate in vivo viral spread through its role in cell migration.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE130665).

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regulation of Ca^{2+} release is critical for viral DNA synthesis and the production of infectious progeny (33, 34). The HCMV immediate-early (IE) protein, pUL37x1, is required for the mobilization of Ca^{2+} from smooth endoplasmic reticulum to the cytosol (34). The Ca^{2+}-dependent protein kinase, PKCα, is activated following infection, which correlates with the efficient accumulation of virions (35). The release of Ca^{2+} during infection might also influence Ca^{2+}-dependent processes that occur in the mitochondria. For example, Ca^{2+} can stimulate aerobic metabolism and enhance ATP production (36, 37). Ca^{2+} can also induce the activity of the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)/5′ AMP-activated protein kinase (AMPK) signaling pathway (38). AMPK activity supports HCMV-induced changes to the infected cell metabolome, and is necessary for HCMV DNA synthesis and the expression of viral late genes (39, 40). Additionally, cellular phosphoinositide 3-kinase (PI3K) and p38 kinase activities are required for viral DNA replication and the production of infectious progeny (41, 42) and both were shown to be downstream factors of the purinergic receptor signaling axis (43, 44). Finally, since Ca^{2+} can trigger apoptosis, regulating the concentration or localization of intracellular Ca^{2+} may affect the infected cell's sensitivity to apoptotic stimuli (45).

Although the viral pUL37x1 protein has already been implicated in mediating Ca^{2+} release, there may also be other pathways that affect intracellular Ca^{2+} levels during HCMV infection. Therefore, we speculated that purinergic receptor-mediated signaling may work alongside pUL37x1 in regulating Ca^{2+} release into the cytosol, influencing the efficiency of HCMV replication. To test this notion, we used pharmacological agents and small interfering RNA (siRNA) technology coupled with RNA sequencing (RNA-seq) to study the consequences of inhibiting purinergic receptors during HCMV infection. We provide evidence that expression of the P2Y2 and P2X5 receptors are induced not only in HCMV-infected, but also in adenovirus (Ad5)-infected cells. We provide evidence that P2Y2-mediated signaling impacts HCMV gene expression and DNA synthesis. The receptor significantly contributes to the regulation of intracellular Ca^{2+} homeostasis and downstream Ca^{2+}-dependent signaling pathways, and it also impacts motility of infected cells.

Results

HCMV Infection Elevates the Levels of RNAs Encoding Several Members of the Purinergic Receptor Network. To investigate the differential expression of cellular genes during infection, we analyzed our recently published dataset (46) derived by whole RNA sequencing of MRC-5 fibroblasts that were mock-infected or infected with the wild-type AD169 laboratory strain of HCMV (AD*wt*; 3 $TCID_{50}$ per cell). RNA was analyzed at 48 h post-infection (hpi), allowing us to monitor RNAs during the early-late phase of the viral replication cycle (the late phase begins with the onset of viral DNA replication at 24 to 30 hpi; maximal yield of progeny is achieved at about 96 hpi). Several components involved in a purinergic receptor signaling network were expressed at significantly higher levels in infected cells—including RNAs encoding P2Y2, P2X5, ENPP4, and PANX2—and a few others were reduced (*SI Appendix*, Table S1).

To confirm the results obtained from the RNA-seq analysis and extend the findings to a different fibroblast cell population and virus strain, we used qRT-PCR to quantify the levels of transcripts identified by RNA-seq analysis. Human foreskin fibroblasts (HFFs) were infected with the TB40/E-GFP clinical isolate of HCMV at a multiplicity of 3 $TCID_{50}$ per cell, and P2Y2, P2X5, ENPP4, and PANX2 RNAs were again characterized by increased levels at 48 hpi (*SI Appendix*, Table S1).

P2Y2 and P2X5 Exhibit Different Expression Kinetics during Infection. We hypothesized that overexpressed transcripts might identify cellular functions that significantly impact the viral replication cycle, so we focused further analysis on the up-regulated purinergic receptors. To examine the kinetics of P2Y2 and P2X5 expression, HFFs were infected with TB40/E-GFP at a multiplicity of 3 $TCID_{50}$ per cell, and samples were collected after various time intervals. P2Y2 and P2X5 RNAs were assayed by qRT-PCR, and the receptor proteins were assayed by Western blotting. P2Y2 RNA was elevated at 24 hpi and increased as the infection progressed (Fig. 1*A*), and P2X5 transcript levels increased up to 48 hpi and then decreased later in infection (Fig. 1*B*). Protein levels tracked RNA levels (Fig. 1*C*). P2Y2 protein substantially increased in abundance at 48 hpi and stayed elevated through 120 hpi, the last time point assayed. P2X5 protein levels increased between 24 and 48 hpi, and then markedly decreased later in infection.

To determine whether other viruses also induce expression of P2Y2 and P2X5, we tested the levels of receptor RNAs following infection with 2 other DNA viruses (herpes simplex 1 [HSV1] and Ad5), as well as 3 RNA viruses (influenza [IAV], Zika virus [ZIKV], and HCV). Of these viruses, only Ad5-infected cells exhibited increased expression of P2Y2 (7.8×) and P2X5 (3.4×) compared to uninfected cells (Fig. 1*D*), suggesting that purinergic receptor up-regulation is neither characteristic nor specific to the herpesvirus family.

HCMV IE1 Protein Induces P2Y2, but Not P2X5. To test whether HCMV gene expression is needed to modulate expression of the purinergic receptors, we used UV-irradiated TB40/E-GFP virus that can bind to and enter cells but cannot express its genes. HFFs were infected with the untreated virus or UV-irradiated virus at a multiplicity of 3 $TCID_{50}$ per cell, samples were collected at 24 and 48 hpi, and transcript levels were quantified by qRT-PCR. Compared to cells infected with untreated TB40/E virus, those infected with the UV-irradiated virus exhibited 90% lower expression of P2Y2 RNA (Fig. 1*E*), but 40% higher expression of P2X5 (Fig. 1*F*). Therefore, our results indicate that virion binding and entry is not sufficient to produce the increased levels of P2Y2 transcript normally seen during infection, whereas the lack of viral gene expression does not prevent the up-regulation of P2X5 expression.

Based on the kinetics of P2Y2 expression, we speculated that IE and early (E) viral protein expression could induce P2Y2 accumulation. To test this hypothesis, we focused on the viral IE1 and IE2 proteins, which are known to be promiscuous transcriptional activators (47, 48). MRC5 fibroblasts were engineered to contain tetracycline-inducible GFP, IE1, IE2, or IE1+IE2 cDNAs (46). Only IE1-expressing cells (MRC5-IE1) were characterized by a strong induction of P2Y2 transcripts at 72 and 96 h post-doxycycline treatment (Fig. 2*A*), which translated also into a 4.4-fold higher P2Y2 protein level when compared to MRC5-GFP (Fig. 2*B*). Even though P2Y2 transcripts levels were only minimally elevated in MRC5-IE2 (fold-change 1.29 ± 0.22) and MRC5-IE1/IE2 (fold-change 1.50 ± 0.51) at 72 h after doxycycline treatment (Fig. 2*A*), an ∼2-fold increase of P2Y2 protein were detected in these cells when compared to MRC5-GFP (Fig. 2*B*). We speculate that in case of IE1 the effect on P2Y2 levels is most likely transcriptional (47, 48); however, IE2 may regulate P2Y2 RNA stability, efficiency of its translation, or indirectly other factors with transcriptional activity (49, 50). We detected only a minimal effect of IE1 and IE2 expression on P2X5 levels (Fig. 2*A*). Of note, cells expressing both IE1 and IE2 proteins did not express P2Y2 (Fig. 2*A*) or had lower P2Y2 protein levels (Fig. 2*B*) compared to MRC5-IE1, suggesting that IE2 protein inhibits P2Y2 expression. IE2 clearly doesn't inhibit expression of the receptor in the context of infection (Fig. 1), but could, of course, limit the extent of its induction. As elevated levels of CXCL10 were reported in IE1-expressing cells (51), we monitored CXCL10 expression in our IE1 and IE2-expressing cells. Both IE1 and IE2 expressed separately and in a combination induced CXCL10 expression without any noticeable inhibitory effect caused by IE2 in double-expressing cells (Fig. 2*C*). Additionally, we have previously shown that IE1 and IE2 expressed in this model cooperate to activate expression of different target genes (46). IE1 and IE2 proteins are known to have distinct functions (52), but at this point the mechanistic basis for the inhibitory effect of IE2 on P2Y2

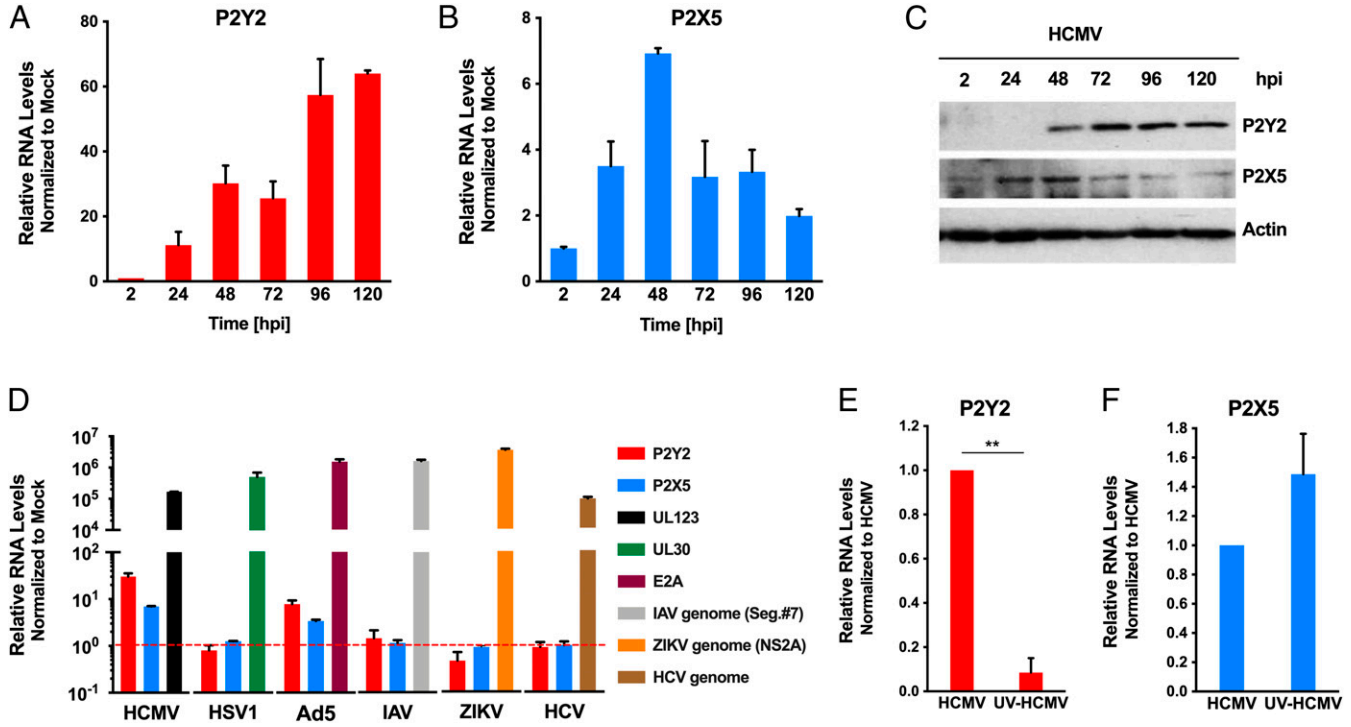

Fig. 1. P2Y2 and P2X5 RNA and protein levels are differentially modulated during HCMV infection. HFFs were infected with TB40/E-GFP (3 $TCID_{50}$ per cell) or mock-infected. RNA and protein samples were collected at various times after infection. (*A* and *B*) P2Y2 and P2X5 RNA accumulation following infection. Transcripts were quantified by qRT-PCR using GAPDH RNA as an internal control. Results are shown as fold-change compared to mock-infected cells. Data are presented as a fold-change mean ± SD for 3 independent experiments. (*C*) P2Y2 and P2X5 protein accumulation following infection. Proteins were separated by SDS/PAGE and subjected to Western blot analyses using antibodies specific for P2Y2 and P2X5. Actin was monitored as a loading control. (*D*) P2Y2 and P2X5 RNA accumulation following infection with different viruses. Fibroblasts were infected with HCMV (3 $TCID_{50}$ per cell), HSV1 (3 $TCID_{50}$ per cell), Ad5 (10 FFU per cell), FLU (3 $TCID_{50}$ per cell), or ZIKV (10 PFU per cell), and Huh7 cells were infected with HCV (1 $TCID_{50}$ per cell). RNA samples were collected at 9 hpi (HSV) or 24 hpi (all other viruses). P2Y2- and P2X5-specific primers were used for qRT-PCR analysis. Viral infection was controlled by probing for the presence of viral transcripts: UL123 (HCMV), UL30 (HSV1), E2A (Ad5), or viral genomes: IAV and ZIKV. GAPDH RNA was monitored as an internal control. Data are presented as a fold-change mean ± SD for 3 independent experiments. A red dotted line depicts normalized RNA levels in mock-infected cells. (*E* and *F*) UV-irradiated HCMV failed to increase P2Y2 but did increase P2X5 RNA levels. Untreated or UV-irradiated TB40/E virus was applied to HFFs (3 $TCID_{50}$ per cell). Samples were collected at 48 hpi (P2Y2) or 24 hpi (P2X5), and transcripts were quantified by qRT-PCR. GAPDH was assayed as an internal control. Data are presented as a fold change mean ± SD for 3 independent experiments. $^{**}P < 0.01$.

expression outside the context of infection is unclear. IE1 protein expression alone induces P2Y2 RNA levels by a factor of about 16 (Fig. 2*A*), whereas infection induces the RNA by a factor of >50 (Fig. 1*A*). It is possible that the different quantitative effects result from different levels or modification states of IE1 protein when it is expressed alone versus within infected cells. Alternatively, an additional unidentified viral protein might cooperate with IE1 to fully induce accumulation of P2Y2.

Taken together, the data confirm that HCMV and Ad5 infections in fibroblasts cause an increase in the expression of the cellular purinergic receptors P2Y2 and P2X5; and, in the case of P2Y2, the HCMV IE1 protein contributes importantly to its induction.

P2Y2 and P2X5 Receptors Have Opposite Effects on HCMV Yield. To determine whether the up-regulated expression of purinergic receptors influences HCMV replication, we assessed the effects of inhibiting their expression or activity on the production of infectious progeny.

First, we employed siRNAs to knock down their expression. Analysis by qRT-PCR showed that siP2Y2 reduced P2Y2 RNA expression to about 15% or 8% of its normal level and siP2X5 reduced P2X5 expression to about 10% or 30% of normal at 48 or 96 hpi (Fig. 3*A*). To test for an effect on virus yield, HFFs were transfected with siRNAs for 24 h before HCMV infection at a multiplicity of 3 $TCID_{50}$/mL. At 96 hpi, the medium was collected and viral titer assayed. Knockdown of the P2Y2 receptor reduced the virus yield to about 10%, whereas knockdown of P2X5 increased the yield to almost 400%, compared to virus in the medium of cells treated with a nonspecific, scrambled siRNA (siSc) (Fig. 3*B*).

We next employed pharmacological perturbations to confirm the roles of these receptors during HCMV infection. Kaempferol is a selective P2Y2 receptor antagonist (15, 53), and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS) has ~10-fold higher affinity for blocking P2X5 than other P2X family members (19). We used kaempferol and PPADS at 50 μM, because the drugs are commonly used to treat fibroblasts at concentrations between 20 and 100 μM (54–57). When uninfected fibroblasts were treated with either drug at doses ranging from 0 to 400 μM, no toxicity was evident (*SI Appendix*, Fig. S1). To test for an effect of virus yield, HFFs were pretreated with drug for 1 h prior to TB40/E-GFP infection at a multiplicity of 3 $TCID_{50}$ per cell. After allowing 2 h for virus entry, cells were washed and supplemented with medium containing either kaempferol or PPADS. Medium with drug was replaced every 24 h until samples were collected at 96 hpi and assayed for infectious virus. Kaempferol decreased the viral yield by >99.8%, whereas PPADS nearly tripled the percent of $IE1^+$ cells in the reporter assay (Fig. 3*C*).

These experiments demonstrate that elevated P2Y2 receptor activity facilitates infection whereas the P2X5 receptor plays an inhibitory role.

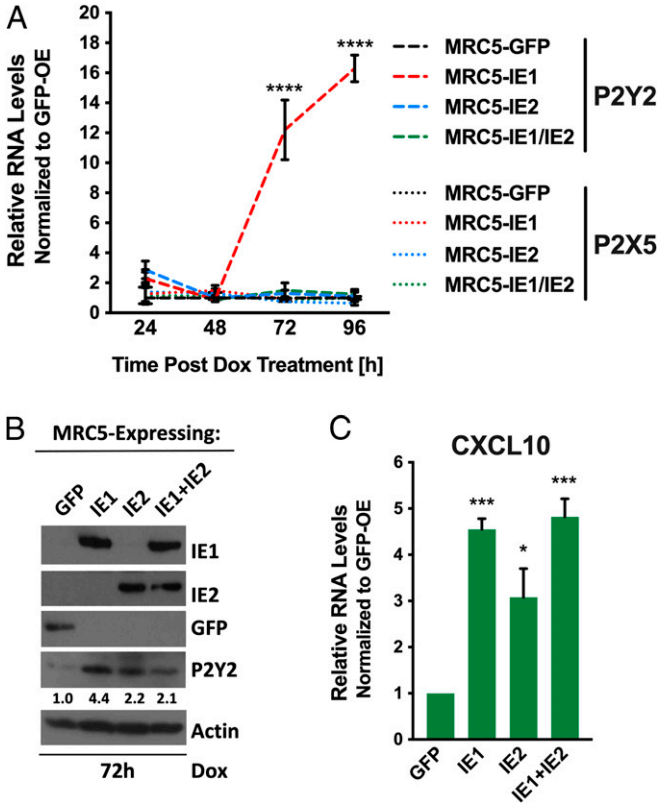

Fig. 2. Expression of P2Y2, but not P2X5, is induced by the viral IE1 protein. Tetracycline-inducible IE1 and/or IE2 MRC-5 cells were treated with doxycycline. (*A*) RNA accumulation following doxycycline treatment. RNA samples were collected at indicated times and qRT-PCR was performed using P2Y2- and P2X5-specific primers. GAPDH was used as an internal control. Data are presented as a fold change mean ± SD for 3 independent experiments. (*B*) Protein accumulation following doxycycline treatment. Protein samples were collected at 72 h after the initial doxycycline treatment and protein levels were analyzed by Western blotting using with anti-GFP, anti-IE1, anti-IE2, or anti-P2y2 antibodies. Actin was used as a loading control. P2Y2 levels were determined by densitometry and relative signal intensities, determined by densitometry and normalized to actin, are depicted. (*C*) Induction of IE1 and IE2 expression induces CXCL10 expression. RNA samples were collected at 24 h after the initial doxycycline treatment and qRT-PCR was performed using CXCL10-specific primers. GAPDH was used as an internal control. Data are presented as a fold change mean ± SD for 3 independent experiments. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$.

Inhibiting P2Y2 or P2X5 Does Not Affect the Efficiency of Viral Entry into Fibroblasts. P2Y2 and P2X5 are cell surface receptors, so it is possible that they influence the initial entry of the virus into fibroblasts. Although this notion does not fit well with the kinetics of receptor expression following HCMV infection (Fig. 1), P2Y2-mediated signaling was shown to be involved in the entry of HIV-1 at the virus–host membrane fusion step (15). Therefore, we tested for an effect of P2Y2 and P2X5 on viral entry using pharmacological inhibitors or siRNAs. HFFs were either pretreated with kaempferol (50 μM), PPADS (50 μM), or solvent control for 1 h, or transfected with siP2Y2, siP2X5, or siSc as a control for 24 h. Then, the cells were infected with TB40/E-GFP virus (1 $TCID_{50}$ per cell) for 1 h at 4 °C, incubated at 37 °C for 1 h and washed with citrate buffer. Viral entry was assayed by either immunostaining for IE1 protein at 24 hpi or qPCR quantification of intracellular viral DNA at 1 hpi at 37 °C. Treatment with either of the drugs did not significantly alter the percentage of cells expressing IE1 protein (Fig. 3*D*), and siRNAs did not change levels of viral DNA (Fig. 3*E*) compared to controls. We conclude that inhibiting P2Y2 and P2X5 activity or expression does not affect HCMV entry into susceptible fibroblasts.

P2Y2 Inhibition Reduces Accumulation of Viral RNAs, Proteins, and DNA. Our data suggested that P2Y2 up-regulation supports HCMV infection, whereas increased P2X5 expression might reflect a cellular defensive response to the infection. Since P2Y2 expression was directly linked to viral gene expression (Figs. 1*E* and 2), required for efficient viral production (Fig. 3) and, as noted above, known to control cellular processes altered by infection, we focused further analysis on the role of P2Y2 during infection.

We tested the accumulation of viral RNAs at 96 hpi by RNA-seq analysis of P2Y2-deficient cells compared to control fibroblasts (58). All viral transcripts monitored were modestly reduced (ranging from 1.2- to 2.2-fold) in P2Y2-deficient cells (Fig. 4*A*). Of note, 2 HCMV transcripts, UL146 and UL147, encoding the viral CXCL1 (vCXCL1) and vCXCL2 chemokines (59), respectively, were affected to the greatest extent. Similarly to viral RNAs, the expression of all viral proteins tested was reduced in P2Y2-deficient cells, but to a somewhat greater extent, with the exception of pUL37x1, which was reduced several fold at earlier times after infection but was present at nearly normal levels at 96 and 120 hpi (Fig. 4*B*). To confirm the data obtained following P2Y2 knockdown, we tested an effect of kaempferol on viral protein levels. Cells were pretreated with the inhibitor for 2 h before being infected with TB40/E-GFP (3 $TCID_{50}$ per cell). The drug was replaced every 24 h until protein samples were collected at 96 hpi. Similarly to siRNA-mediated depletion of P2Y2, kaempferol pretreatment reduced the level of IE2 and all early and late HCMV proteins tested (Fig. 4*C*). Curiously, unlike during siRNA-based P2Y2 knockdown, kaempferol pretreatment did not markedly reduce the level of IE1 protein. Kaempferol with other flavonoids was suggested to be a noncompetitive antagonist of P2Y2 receptor (53). Perhaps the divergent effects of P2Y2 siRNA and kaempferol on IE1 protein levels result from kaempferol preferentially affecting P2Y2 signaling functions, whereas siRNA-mediated reduced P2Y2 levels may additionally block the consequences of its interactions with other cell surface receptors, as for EGFR in prostate cancer cells (60). Quantitative differences between viral RNAs and proteins might result from changes to translational initiation (61) and protein stability (62), both of which are actively modulated by HCMV during infection. In sum, our results indicate that P2Y2 contributes to HCMV gene expression in fibroblasts after viral entry.

We also tested whether P2Y2 impacted viral DNA accumulation. Total DNA was isolated from HCMV-infected siSc- or siP2Y2-treated cells at 96 hpi, and viral DNA copy number was measured by qPCR. There was about 10-fold less viral DNA in P2Y2-deficient cells than in infected control cells (Fig. 4*D*). We also compared the amount of DNase I-resistant viral DNA (a proxy for virion DNA) in the medium of siP2Y2-treated cells as compared to siSc-treated controls. There were ∼3-fold fewer copies of viral DNA released from P2Y2-deficient cells compared to control cells (Fig. 4*E*). Interestingly, the decrease in infectious virus yield from P2Y2-deficient cells was greater (∼10-fold) (Fig. 3*D*) than the decrease seen for cell-free viral DNA (∼3-fold) (Fig. 4*E*). To test whether progeny released from P2Y2 knockdown cells are less infectious, the ratios of viral DNA copy number to infectious units were calculated. The samples collected from P2Y2-deficient cells were less infectious (∼4× more particles per infectious unit) than samples collected from siSc-treated cells (Fig. 4*F*), indicating that there are not only fewer virions released from P2Y2-deficient cells, but their infectivity is also compromised.

P2Y2 supports the accumulation of intracellular viral RNA, protein, and DNA and, ultimately, the efficient production of infectious extracellular virions.

P2Y2 and pUL37x1 Cooperate to Regulate Intracellular Ca^{2+} during Infection. Viral DNA synthesis and the production of infectious progeny are reduced by drugs that disrupt Ca^{2+} homeostasis in

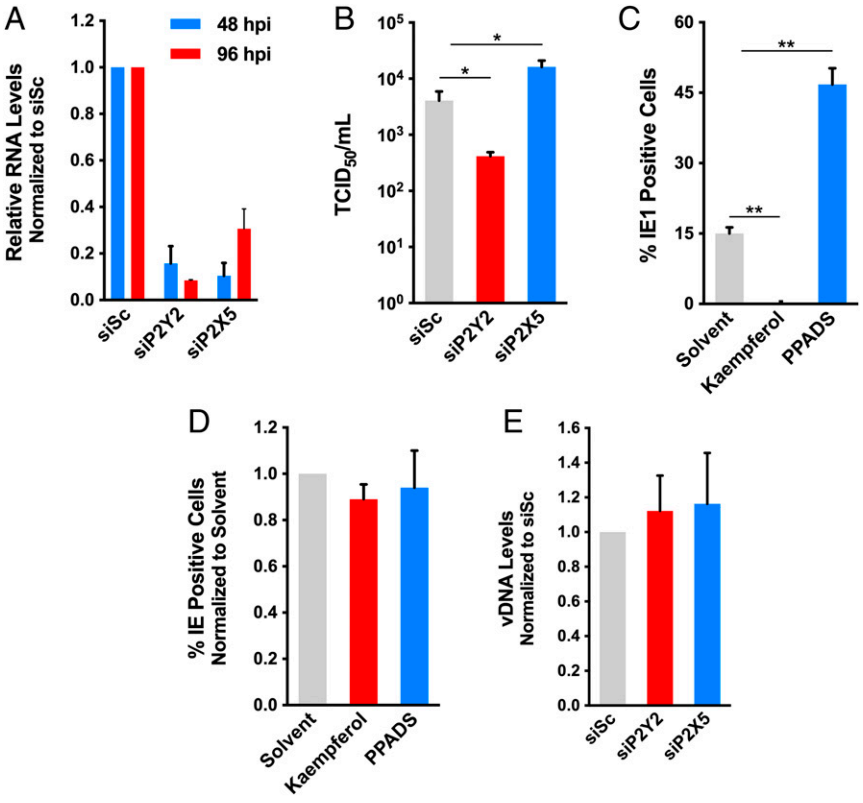

Fig. 3. P2Y2 and P2X5 act after viral entry to modulate HCMV yield. (*A*) siRNAs reduce P2Y2 and P2X5 RNA levels. HFFs were transfected with siP2Y2, siP2X5, or siSc as a control. After 24 h, cells were infected with TB40/E-GFP (3 $TCID_{50}$ per cell). RNA samples were collected at 48 and 96 hpi and qRT-PCR was used to measure levels of P2Y2 and P2X5 transcripts. GAPDH was used as an internal control. Data are presented as fold-change mean ± SD for 3 independent experiments. (*B*) siRNAs targeting P2Y2 or P2X5 modulate virus yield. HFFs were transfected with siP2Y2, siP2X5, or siSc as a control. After 24 h, cells were infected with TB40/E-GFP (3 $TCID_{50}$ per cell). Virus yields were determined at 96 hpi. Results are presented as an average $TCID_{50}$/mL ± SD for 3 independent experiments. (*C*) Drugs targeting P2Y2 or P2X5 modulate virus yield. HFFs were infected with TB40/E-GFP virus (3 $TCID_{50}$ per cell) or mock-infected for 2 h and treated with either kaempferol (50 μM) or PPADS (50 μM). The drugs were replaced every 24 h until media samples were collected at 96 hpi. Viral yields were determined by monitoring expression of IE1 protein at 24 h after media transfer into a reporter plate. Results are presented as average percent of $IE1^+$ cells ±SD for 3 independent experiments. (*D* and *E*) HFFs were either (*D*) pretreated with kaempferol (50 μM), PPADS (50 μM), or solvent control for 1 h, or (*E*) transfected with siP2Y2, siP2X5, or siSc as a control for 24 h. Then, cells were infected with TB40/E-GFP virus (1 $TCID_{50}$ per cell) for 1 h at 4 °C, incubated at 37 °C for 1 h, and washed with citrate buffer. Viral entry was assayed by either (*D*) immunostaining for IE1 protein at 24 hpi or (*E*) qPCR quantification of intracellular viral DNA at 1 hpi. Data are presented as a fold change mean ± SD for 3 independent experiments. $*P < 0.05$, $**P < 0.01$.

the endoplasmic reticulum (33) and by loss of the HCMV IE protein, pUL37x1, which mobilizes Ca^{2+} from the smooth endoplasmic reticulum to the cytosol (34). P2Y2 also mobilizes Ca^{2+} (12), and its knockdown or inhibition reduced viral DNA accumulation (Fig. 4*D*) and yield (Fig. 3 *B* and *C*). However, accumulation of protein encoded by UL37x1 exhibited reduced P2Y2 dependence compared to some other viral gene products (Fig. 4 *B* and *C*). Therefore, we investigated the effects of inhibiting P2Y2 activity on intracellular Ca^{2+} levels during infection. To do so, fibroblasts were pretreated for 2 h with the P2Y2 inhibitor, kaempferol, or solvent as a control, and then mock-infected or infected with either wild-type (AD*wt*) or pUL37x1-deficient (AD*sub*UL37x1) virus. Ca^{2+} levels were measured at 20 hpi using the fluo-4 AM calcium assay. As shown previously (34), wild-type virus, but not pUL37x1-deficient virus, caused an ∼2.5-fold increase in cytosolic Ca^{2+} levels compared to mock-infected cells (Fig. 5*A*). Kaempferol completely blocked the increase in Ca^{2+} caused by infection with wild-type virus and had no significant effect on Ca^{2+} levels within cells infected with pUL37x1-deficient virus.

To test if the lack of pUL37x1 expression affects P2Y2 levels, HFFs were infected with either AD*sub*UL37x1 or its wild-type parent. Samples were collected at 24 hpi, and transcripts were quantified by qRT-PCR. P2Y2 RNA accumulated to similar levels in cells infected with either virus (*SI Appendix*, Fig. S2*A*). In control experiments, the same level of UL123 RNA accumulated up to 24 hpi, confirming similar infection dynamics when the 2 viruses were used (*SI Appendix*, Fig. S2*B*); and UL37x1 RNA expression was evident in wild-type but not in mutant virus-infected cells (*SI Appendix*, Fig. S2*C*). Additionally, indirect immunofluorescence showed that pUL37x1 levels were reduced at 20 hpi in kaempferol- vs. DMSO-treated cells, consistent with the effect of P2Y2 knockdown (Fig. 4*B*), but its cellular localization was not changed (Fig. 5*B*).

To further explore the interaction of P2Y2 and pUL37x1, we investigated the effect of pUL37x1 expression on Ca^{2+} homeostasis in uninfected fibroblasts. For this purpose, we used 2 HFF cell derivatives, UL37x1 (1)-HFF and UL37x1 (2)-HFF, that stably express pUL37x1 (Fig. 5*C*). The cells were treated with kaempferol or solvent control for 1 h, and then Ca^{2+} levels were measured using the fluo-4 AM assay and compared to levels in control cells lacking the viral protein. No significant differences in Ca^{2+} levels were observed between samples (Fig. 5*D*). When pUL37x1-expressing cells were infected with AD*sub*UL37x1, cytosolic Ca^{2+} was increased and, as expected, kaempferol treatment inhibited the increase (Fig. 5*E*). We interpret this experiment to indicate that pUL37x1 and P2Y2 cooperate to increase intracellular Ca^{2+} levels only in the context of an infected cell or that the increase cannot occur in the absence of the P2Y2 overexpression observed following infection.

In sum, these data demonstrate that P2Y2 and pUL37x1 collaborate to increase intracellular Ca^{2+} levels during HCMV infection.

P2Y2 Alters Cellular RNA Levels and Motility of HCMV-Infected Cells. Because our data demonstrated effects of P2Y2 on viral replication and the regulation of intracellular Ca^{2+} levels, we decided to investigate more broadly the impact of P2Y2 on infected-cell biology by utilizing RNA-seq to monitor global gene expression of cells treated with control or P2Y2 siRNA (58). The differentially expressed cellular genes ($q < 0.05$) in mock- or HCMV-infected HFF cells treated with either siSc or siP2Y2 were analyzed using the NetworkAnalyst platform (63) to create a list of gene sets enriched with P2Y2-regulated genes based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) (64, 65) database. This analysis indentified several cell motility-associated gene sets that were significantly enriched in the KEGG database (focal adhesion [q = 2.05e-7], extracellular matrix-receptor interaction [q = 6.64e-5], pathways in cancer [q = 6.64e-5], regulation of actin cytoskeleton [q = 7.04e-3], adherens junction [q = 3.72e-2]) and in the gene ontology:biological process database (cellular protein complex disassembly [q = 5.43e-8], regulation of cell migration [q = 1.68e-5]). The

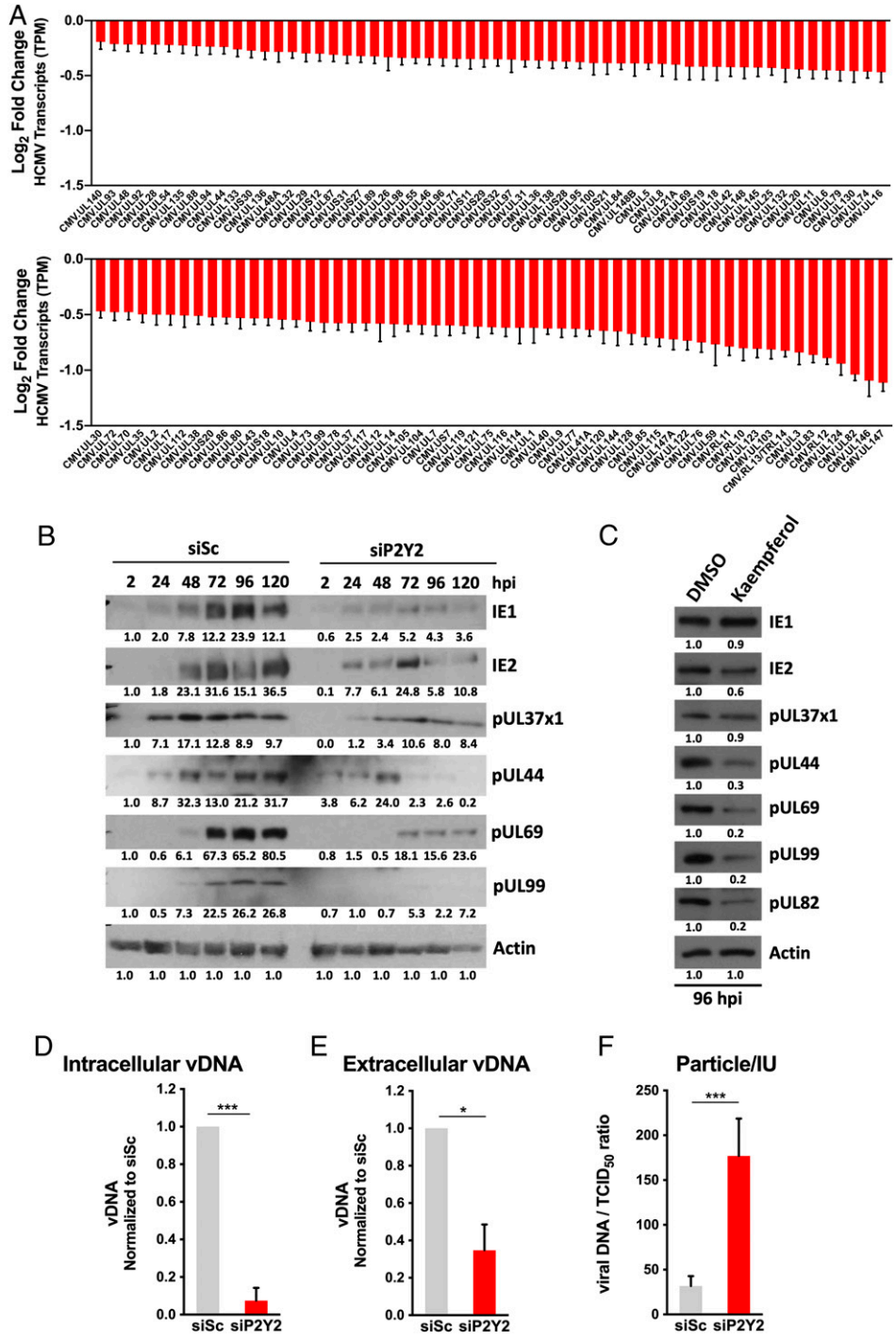

Fig. 4. P2Y2 receptor affects viral RNA, protein, and DNA accumulation. (*A*) P2Y2 supports the accumulation of viral RNAs. HFFs were transfected with siP2Y2 or siSc as a control. After 24 h, cells were infected with TB40/E-GFP (3 $TCID_{50}$ per cell). HCMV gene expression in terms of transcripts per million reads was computed and normalized across samples ±SE for 3 independent experiments. (*B*) HFFs were transfected with siP2Y2 or siSc as a control. After 24 h, cells were infected with TB40/E-GFP (3 $TCID_{50}$ per cell) or mock-infected. Protein samples were collected after various time intervals. Protein levels were determined by densitometric analysis of the Western blot from a representative experiment and relative signal intensities, normalized to actin, are presented under each gel image. (*C*) HFFs were treated with kaempferol (50 μM) or DMSO as a solvent control for 2 h. Then cells were infected with TB40/E-GFP (3 $TCID_{50}$ per cell). The drug was replaced every 24 h until protein samples were collected at 96 hpi. Protein levels were determined by densitometric analysis of the Western blot from a representative experiment, and relative signal intensities, normalized to actin, are presented under each gel image. (*D*) P2Y2 knockdown reduces intracellular vDNA. HFFs were transfected with siP2Y2 or siSc as a control. After 24 h, cells were infected with TB40/E-GFP virus (3 $TCID_{50}$ per cell) or mock-infected. At 96 hpi, total DNA was isolated and the level of intracellular viral DNA was measured by qPCR. Data are presented as vDNA fold-change mean ± SD for 3 independent experiments. (*E*) P2Y2 knockdown reduces extracellular vDNA. At 96 hpi, total DNA was isolated from the DNase I-treated medium of siSc- or siP2Y2-treated, infected cells and the level of extracellular viral DNA was measured by qPCR. Data are presented as vDNA fold-change mean ± SD for 3 independent experiments. (*F*) P2Y2 knockdown increases the particle-to-infectious unit ratio. The infectivity of virus in media collected at 96 hpi was titered and viral DNA was isolated from virions present in the media and quantified by qPCR to calculate a viral DNA copy number-to-infectious unit ratio. Data are presented as the avarage ratios ±SD for 3 independent experiments. *$P < 0.05$, ***$P < 0.001$.

network of KEGG gene sets enriched with P2Y2-regulated genes is shown in Fig. 6*A*. Additionally, our analysis of P2Y2-regulated genes during HCMV infection using Ingenuity Pathway Analysis (IPA) (66) indentified gene signatures associated with cellular movement (*P* value range 2.84e-07 to 6.01e-42) as the highest ranking among molecular and cellular functions modified by P2Y2 in HCMV-infected cells. A closer analysis of genes associated with the cellular movement category determined that the expression of only 3 genes was changed in uninfected cells treated with siP2Y2, perhaps due to the low P2Y2 expression. In contrast, expression of a substantial number of these genes was altered in HCMV-infected cells and affected by P2Y2 siRNA-treatment (Fig. 6*B*). Of note, 82% of genes, associated with the cellular movement and differentially expressed in infected cells, had their expression altered by siP2Y2 (Fig. 6*B*). To expand our investigation of P2Y2-regulated gene expression, we used IPA to focus on those genes with altered expression resulting from inhibition of P2Y2 function. Infected-cell P2Y2-regulated genes were found to overlap with several cell motility-associated pathways (regulation of the epithelial-mesenchymal transition pathway, actin cytoskeleton signaling, FGF signaling) and metabolic pathways (glutamate receptor signaling, cAMP-mediated signaling, glucose-6-phosphate [G6P] signaling pathway, and calcium signaling) (*SI Appendix*, Fig. S3).

HCMV infection modulates the movement of multiple cell types (67–71), a phenotype important in both HCMV spread and latency (67, 72). Given the impact of P2Y2 knockdown on cell motility-associated gene signatures, we asked if P2Y2 could influence HCMV-infected epithelial cell motility as it has previously been shown to do for uninfected corneal epithelial cells treated with UTP (73). A transwell migration assay (74) determined that P2Y2 knockdown did not affect the motility of uninfected epithelial cells, but had a strong inhibitory effect (~60% decrease) on the motility of HCMV-infected cells (Fig. 6*C*). A wound-healing assay (75) revealed that P2Y2-deficient, HCMV-infected cells were significantly slower in closing wounds compared to infected cells with the wild-type levels of P2Y2 (Fig. 6*D*). Overall, our results demonstrate that P2Y2 robustly regulates cell motility following HCMV infection.

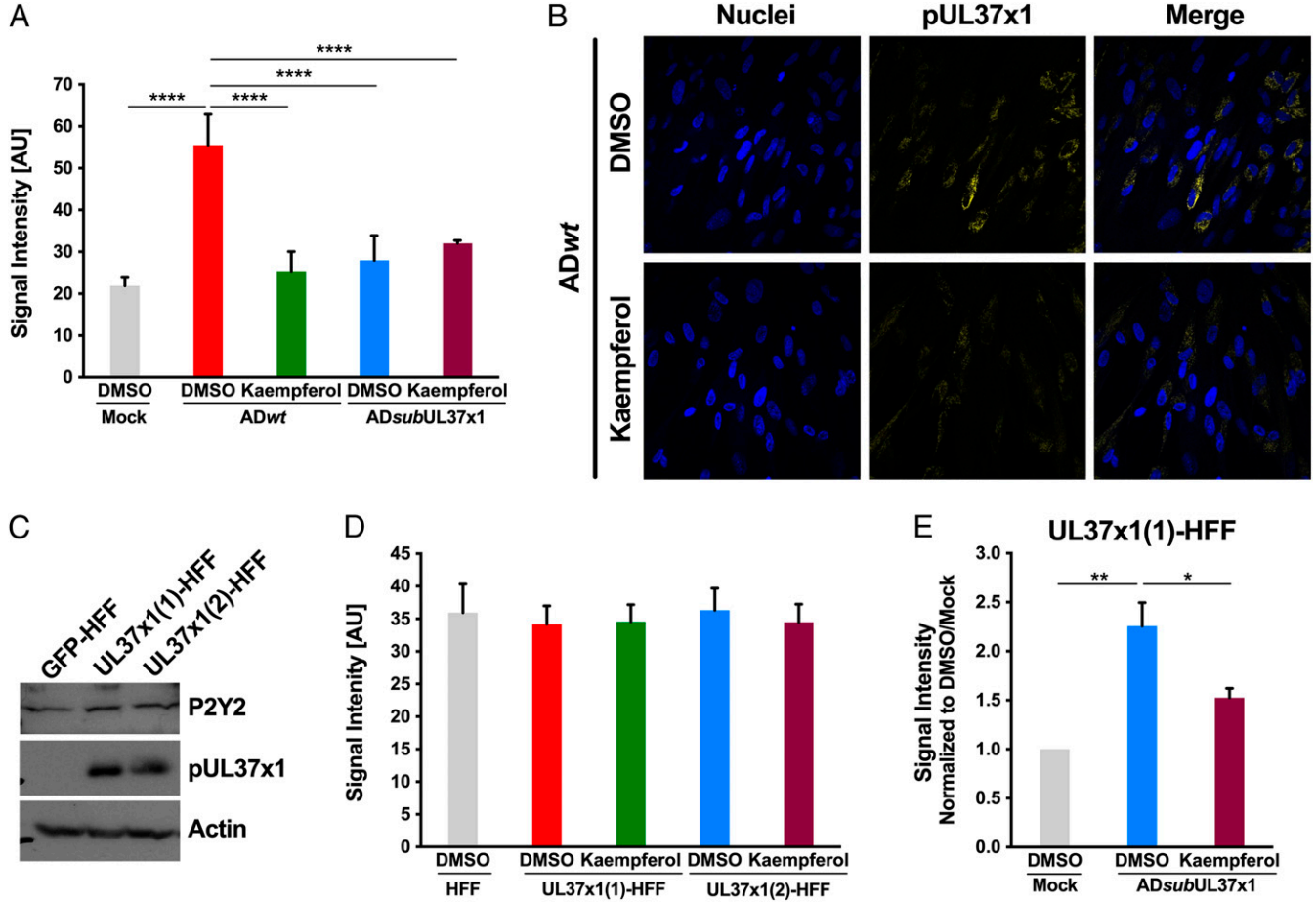

Fig. 5. P2Y2 regulates the increase in cytosolic Ca^{2+} following HCMV infection. (*A*) Effects of kaempferol on Ca^{2+} levels. HFFs were treated with kaempferol (50 μM) or solvent control and then infected with AD*wt* or AD*sub*UL37x1 (1 $TCID_{50}$ per cell) or mock-infected. Ca^{2+} levels were assayed at 20 hpi. Results are presented as the mean intensity of fluorescent signal in arbitrary units (AU) ± SD for 3 independent experiments. (*B*) Kaempferol does not alter pUL37x1 localization. Cells were treated with kaempferol (50 μM) or solvent control and then infected with AD*wt* (1 $TCID_{50}$ per cell). At 20 hpi, cells were fixed and stained with pUL37x1-specific antibody (yellow). Nuclei were counterstained with DAPI dye (blue). (Magnification: 60×.) (*C*) pUL37 overexpression in HFF cells. Protein samples were collected from 2 clones of HFF fibroblasts expressing pUL37x1, UL37x1 (1)-HFF, and UL37x1 (2)-HFF, and control cells expressing GFP (GFP-HFF). Proteins were assayed by Western blot analysis was performed using antibodies recognizing P2Y2, pUL37x1 and actin as a loading control. (*D*) Kaempferol does not alter Ca^{2+} levels in pUL37x1 overexpressing cells. Wild-type HFF cells or HFF cells overexpressing pUL37x1 [UL37 × 1 (1)-HFF and UL37x1 (2)-HFF] were treated with kaempferol (50 μM) or solvent control. Ca^{2+} levels were assayed 1 h later, and results are presented as the mean intensity of fluorescent signal in AU ± SD for 3 independent experiments. (*E*) AD*sub*UL37x1 infection alters Ca^{2+} levels in pUL37x1-expressing cells. UL37x1 (1)-HFF cells were treated with kaempferol (50 μM) or solvent control and then infected with AD*sub*UL37x1 (1 $TCID_{50}$ per cell) or mock-infected. Ca^{2+} levels were assayed at 20 hpi. Data are presented as a fold change mean of the intensity of fluorescent signal ±SD for 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$.

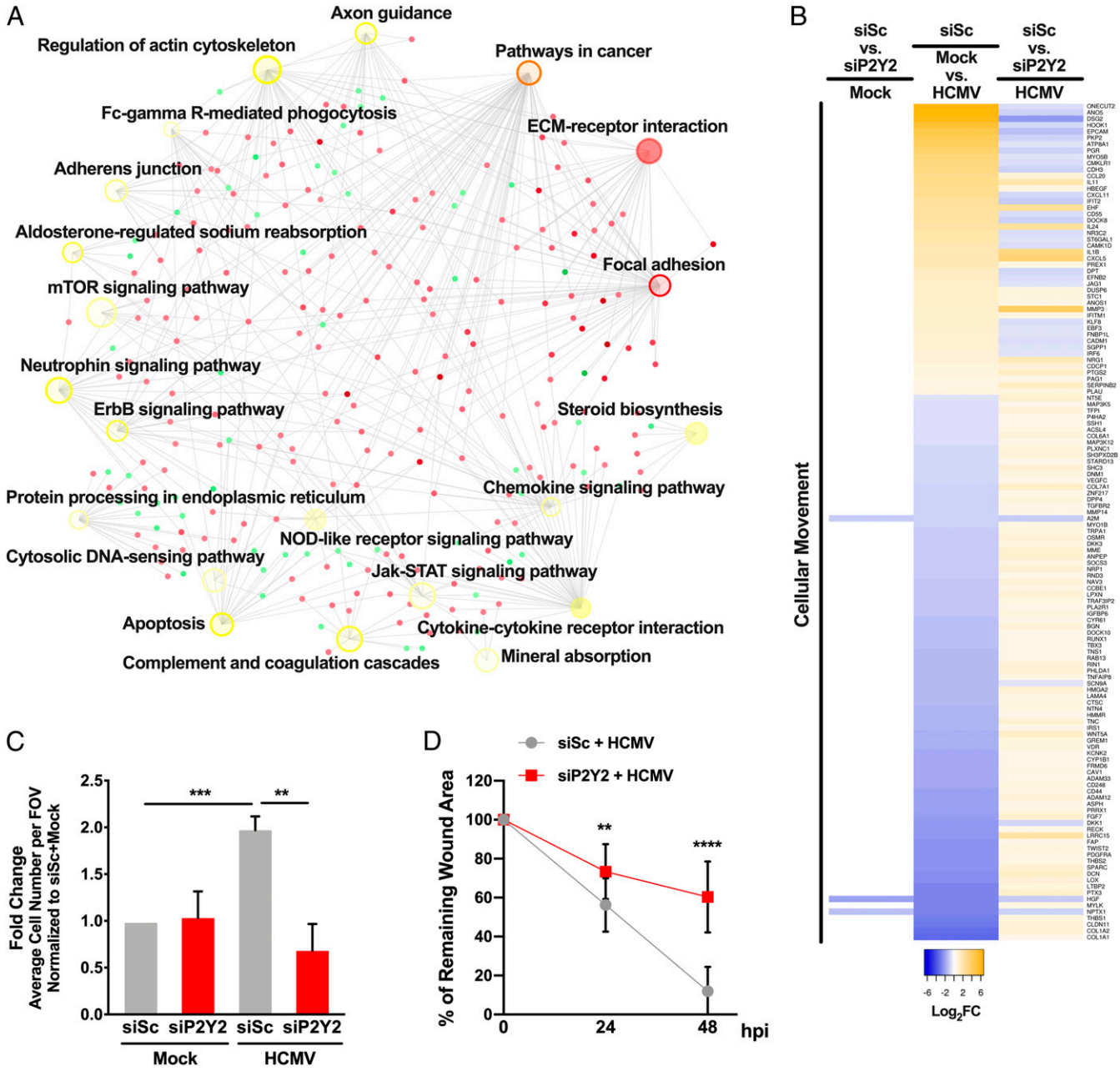

Fig. 6. P2Y2 affects motility of HCMV-infected cells. Fibroblasts were transfected with NT siRNA or P2Y2 siRNA, and 24 h later the transfected cells were mock-infected or HCMV-infected (TB40/E-GFP) at 1 $TCID_{50}$ per cell. At 96 hpi, RNA was isolated and analyzed using RNA sequencing. (*A*) The NetworkAnalyst platform was used to analyze the set of cellular genes differentially expressed ($q < 0.05$) in HCMV-infected, NT siRNA- versus P2Y2 siRNA-transfected HFFs. The network of the KEGG gene sets significantly enriched ($P < 0.05$) in P2Y2-regulated genes with all genes involved is presented. Gene nodes (open, bigger circles) are colored based on the calculated *P* value of their network connectivity (red color indicates lower *P* value and yellow color indicates higher *P* value) and interconnected individual genes (closed, smaller circes) are colored based on their fold-change expression (red color indicates up-regulated genes and green color indicates down-regulated genes). (*B*) The IPA platform was used to analyze the set of cellular genes differentially expressed (fold-change >1.5; $q < 0.05$) in mock- or HCMV-infected, NT siRNA- versus P2Y2 siRNA-transfected HFFs. Predicted molecular functions regulated by P2Y2-dependent genes were identified. Based on the IPA analysis, the heatmap was generated from P2Y2-dependent genes showed a significant overlap with gene sets associated with a cellular movement. Each column represents separate differential gene-expression comparison. Genes are colored based on their fold-change expression in indicated comparisons. (*C*) P2Y2 modulates migration of HCMV-infected ARPE-19 cells in a transwell assay. ARPE-19 cells were transfected with NT siRNA or P2Y2 siRNA 24 h before mock or HCMV (TB40-epi) infection at 3 $TCID_{50}$ per cell. After 3 hpi, cells were transferred onto transwell inserts. Migrated cells were washed, fixed, and stained with Crystal violet. The graph presents a fold-change mean ± SD for 3 independent experiments, based on the number of cells migrated through a transwell per a field-of-view (FOV). (*D*) P2Y2 affects wound closure of HCMV-infected ARPE-19 cells. ARPE-19 cells were transfected with siSc or siP2Y2 24 h before mock or HCMV (TB40-epi) infection at 3 $TCID_{50}$ per cell. At 3 hpi, wounds were created and closure was monitored at indicated times. The graph presents data as a mean percent of remaining wound area ±SD for 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

P2Y2 Regulates the PI3K/AKT Signaling Pathway during HCMV Infection. The family of P2Y receptors are known to regulate a broad range of cellular processes (76, 77), including P2Y2-mediated activation of PI3K, which in turn phosphorylates and activates Akt (78). PI3K-regulated Akt, a known convergence point for multiple upstream cellular signals (79), is modulated in many herpesvirus infections (80). Activation of the pathway is important in HCMV replication (41, 81), and serves to block apoptosis of infected cells (41, 81). Our RNA-seq analysis of P2Y2-regulated gene expression during HCMV infection indicated that ErbB4 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4) signaling is affected (*SI Appendix*, Fig. S4). ErbB4 belongs to the epidermal growth factor receptor (EGFR) family, whose members have important roles in cell proliferation, differentiation, apoptosis, and migration (82). ErbB4 via PI3K/Akt signaling promotes gastric cell proliferation (83) and supports mesenchymal stem cell characteristics (84). Therefore, we tested whether P2Y2 modulates the PI3K/AKT signaling pathway during HCMV infection. Cells were AD*wt*-, AD*sub*UL37x1-, or mock-infected and treated with kaempferol or solvent as a control. Western blot analysis was used to monitor levels of phosphorylated Akt at S473 (rapamycin complex 2 [mTORC2]-dependent) and T308 (phosphoinositide-dependent protein kinase 1 [PDK1]-dependent) that are needed for a full Akt activation (79). Compared to mock-infected cells, AD*wt*- and AD*sub*UL37x1-infected cells showed increased levels of phosphorylated Akt at both positions, and in both instances kaempferol strongly decreased the phosphorylation (Fig. 7). These data indicate that P2Y2, but not pUL37x1, is important for the full activation of Akt in infected cells and suggests that the strong effect of P2Y2 on the HCMV life cycle results in part from its regulation of the PI3K/Akt and the mTORC2 signaling axes.

Discussion

There is a growing body of data suggesting that purinergic receptor family members play important roles during infections (15, 24–30). Specifically for viral infections, the P2X or P2Y receptors have been shown to function in entry of HBV (85, 86), HCV (22), HDV (85, 87), and HIV (15, 88, 89).

In the case of HCMV infection, changes in the levels of purinergic receptors have been reported previously with the P2Y5 receptor expression elevated in latently infected granulocyte-macrophage progenitors (90) and P2Y1, P2Y2, and P2X7 receptors elevated in infected endothelial cells (30). However, the role of purinergic receptors in HCMV infection has not been clear. Here we show that the level of P2Y2 and P2X5 receptors is induced during HCMV (Fig. 1 *A*–*D*) and adenovirus infection (Fig. 1*D*). Given the role of P2Y2 in Ca^{2+} homeostasis following HCMV infection (Fig. 5), it is interesting to note that elevated Ca^{2+} has been reported in Ad5-infected cells (91), and the adenoviral glycoprotein E3-6.7K was found to affect cellular Ca^{2+} homeostasis (92). Conceivably there is a functional interaction between purinergic receptors and E2-6.7K protein.

In our report, we focused on the roles of up-regulated P2Y2 and P2X5 in HCMV-infected cells and found that active viral gene expression, and specifically HCMV IE1 protein, is necessary for elevated P2Y2 accumulation (Figs. 1*E* and 2*A*), but viral entry without viral gene expression suffices for increased accumulation of P2X5 (Figs. 1*F* and 2*A*). Interestingly, P2Y2 expression was positively regulated when IE1 protein was expressed alone, but not in cells expressing both IE1 and IE2 proteins (Fig. 2 *A* and *B*). IE1 and IE2 proteins are known to be promiscuous transcriptional activators (47, 48), but they also possess distinct activities (93). Although IE2 blocked P2Y2 RNA accumulation in response to IE1 when tested outside the context of infection, it clearly does not block within infected cells, where additional viral proteins likely support P2Y2 expression either directly or indirectly.

Even though ATP activates both P2Y2 and P2X5 receptors, they had opposite effects on virus yield. P2Y2-deficient cells produced about ∼90% less virus than cells containing the receptor (Fig. 3 *B* and *C*), showing that P2Y2 supports HCMV replication. In contrast, infection of P2X5-deficient cells resulted in 3- to 4-fold enhanced virus production (Fig. 3 *B* and *C*), showing that P2X5 antagonizes HCMV replication. Our analysis of P2Y2 and P2X5 expression kinetics supports the view that these receptors are independently regulated and have different functions during HCMV infection. While P2Y2 RNA and protein levels gradually increased from 2 to 96 hpi (Fig. 1 *A* and *C*), P2X5 expression increased up to 48 hpi and then decreased afterward (Fig. 1 *B* and *C*). Consistent with the kinetics of P2Y2 and P2X5 expression, P2Y2 and P2X5 did not affect HCMV entry (Fig. 3 *D* and *E*), differentiating the P2Y2 role in HCMV infection from the role in entry described for HIV (15).

How do the purinergic receptors modulate HCMV replication? At this point we can only speculate how the P2X5 receptor antagonizes HCMV. Conceivably, similar to inhibitory effects of the activated P2X7 on replication of Newcastle disease virus, murine leukemia virus, and HSV (94), as well as P2X7 and P2Y6 on replication of vesicular stomatitis virus (94, 95), elevated P2X5 may assist in developing an ATP-mediated antiviral state by inducing expression of IFN-β. HCMV antagonizes numerous intrinsic antiviral responses (96), and the inhibition of P2X5 might be yet another example.

Ca^{2+}-dependent processes play a critical role in HCMV replication (33–35), and the P2Y2 receptor regulates intracellular Ca^{2+} levels (12, 13, 19). Extracellular nucleotides act via the P2Y2 receptor to induce cytosolic Ca^{2+} mobilization from the smooth endoplasmic reticulum in a variety of cell types (97–99). The increase in intracellular Ca^{2+} can be blocked by PLC inhibitors and by low molecular weight heparin, indicating the involvement of IP_3-sensitive intracellular Ca^{2+} stores, which is known to be downstream of P2Y2-mediated signaling (100). P2Y2 works in concert with viral pUL37x1 to elevate cytosolic Ca^{2+} (Fig. 5), and this activity is likely key to its support of viral replicaton. Treating infected cells with drugs that disrupt smooth endoplasmic reticulum Ca^{2+} homeostasis inhibits the production of infectious progeny due to retarded accumulation of late gene products (33), patterns that were observed during HCMV infection in cells deficient in P2Y2 expression (Fig. 4*B*) and cells with chemically blocked P2Y2 function (Fig. 4*C*). Both P2Y2 and pUL37x1 are required to maintain favorable cytosolic Ca^{2+} levels during infection (Fig. 5), but it is not yet clear how they cooperate.

HCMV infection of P2Y2 knockdown cells exhibited a modest reduction in expression of all viral RNAs tested, when assayed by RNA-seq analysis at 96 hpi (Fig. 4*A*). UL44 and UL54 RNAs (Fig. 4*A*) and UL44 protein (Fig. 4*B*) were reduced and, not surprisingly, P2Y2-deficient and kaempferol-treated cells exhibited reduced accumulation of intracellular viral DNA (Fig. 4*D*). In addition to its effect on levels of the viral polymerase, there are additional mechanisms by which the P2Y2 receptor might affect viral DNA replication. Extracellular nucleotides stimulate the PI3K/Akt pathway through P2Y2-mediated signaling (101), a PI3K inhibitor has been shown to decrease viral DNA accumulation (41), and our data suggest that P2Y2 activity regulates activation of Akt via mTORC2- and PDK1-dependent phosphorylations in infected cells (Fig. 7). Even though intracellular Ca^{2+} was found to stimulate Akt activity in different cell types (102, 103), this effect was not dependent on the UL37x1/Ca^{2+} signaling axis (Figs. 5 and 7). Hence, the integrative effect of P2Y2-dependent regulation of the PI3K/mTORC2 and PI3K/PDK1 signaling axes seems to be distinct from its effects on intracellular Ca^{2+} concentrations. Furthermore, the P2Y2-dependent Akt activation within the first 24 hpi expands our understanding of how P2Y2 functions, independently as well as in collaboration with the UL37x1 protein, in regulating biological processes during HCMV infection.

Our transcriptomic analysis also revealed that P2Y2 deficiency had the strongest effect on the expression of UL146 and UL147 (Fig. 4*A*), 2 viral genes encoding vCXCL1 and vCXCL2 (104), respectively. Their ORFs are localized in the UL133-151 region of viral DNA that is found in low-passage HCMV clinical strains,

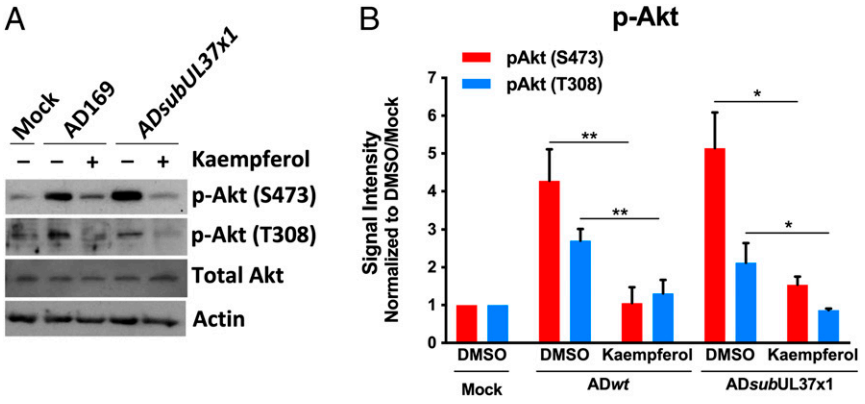

Fig. 7. P2Y2 regulates the phosphorylation state of Akt in HCMV-infected cells. HFFs were treated with kaempferol (50 μM) for 1 h and then infected with AD*wt* or AD*sub*UL37x1 viruses (3 $TCID_{50}$ per cell) or mock-infected, cells were harvested at 24 hpi, and protein levels were determined by a Western blot analysis. (*A*) Results are displayed from a representative Western blot experiment. (*B*) Densitometric analyses of phospho-Akt (S473) and phospho-Akt (T308) signals are shown. Signal values were adjusted based on actin levels. Data are presented as a fold-change mean ± SD for 3 independent experiments. $*P < 0.05$, $**P < 0.01$.

but not in laboratory strain AD169 (105). Interestingly, vCXCL1 was shown to induce Ca^{2+} mobilization and chemotaxis of neutrophils (59) through its interaction with cellular chemokine receptors (106), stimulating us to speculate about a possible functional cooperation between P2Y2, UL37x1, and UL146 in regulating Ca^{2+} homeostasis and efficient HCMV replication.

To further explore the role that the P2Y2 receptor plays in viral infection, we asked what effects P2Y2 deficiency has on the cellular transcriptome following HCMV infection (Fig. 6 *A* and *B* and *SI Appendix*, Fig. S3). A potential complication to interpretation of these results is the fact that P2Y2 knockdown modestly reduces expression of multiple viral genes (Fig. 4 *A*–*C*), which could in turn influence expression of cellular genes. However, several P2Y2-dependent changes observed in infected cells are very likely, at least in part, direct effects of receptor action. Infected cell migration is a case in point. P2Y2 has been shown to influence the movement of multiple cell types. For example, P2Y2 activation is known to increase MCF-7 breast cancer and hepatocellular carcinoma cell migration (13, 107). Specifically, extracellular ATP can activate MAPKs through the P2Y2/PLC/PKC/ERK signaling pathway to induce the translocation of $ERK_{1/2}$ into the nucleus (108). In addition, fibroblasts appear to require PKC activation in order to respond to hyaluronan stimulation with increased locomotion (109). HCMV infection is also known to modulate the movement of multiple cell types (67–71) and our infected-cell data showed a strong enrichment of P2Y2-regulated genes to several gene sets associated with cell motility (Fig. 6 and *SI Appendix*, Fig. S3). P2Y2-deficient, HCMV-infected cells are characterized by significantly reduced transwell migration and wound healing processes compared to control cells (Fig. 6 *C* and *D*).

Metabolism is profoundly changed in cancer cells and is characterized by a high rate of glycolysis, glutamine anaplerosis, and fatty acid synthesis (110–112). Metabolic processes are also critical for effective virus propagation, as they provide necessary energy and building blocks for production of progeny virus (113–115). HCMV not only relies on, but also actively governs host cell metabolism (116–121). Remarkably, HCMV-mediated changes in cellular metabolism are very similar to those seen in tumor cells (116, 122). HCMV infection deregulates nucleotide biosynthesis (123). Our transcriptome analysis also indicates that P2Y2 influences metabolomic processes within infected cells, including glutamate receptor signaling, cAMP-mediated signaling, and G6P signaling pathways, as well as inflammatory responses (*SI Appendix*, Fig. S3), identifying additional avenues for investiging the P2Y2-dependent regulation of cellular biology during viral infection.

PI3K-regulated Akt, a known convergence point for multiple upstream cellular signals (79), including metabolism (124) and cell motility (125), is modulated in many herpesvirus infections (80) and activation of the pathway is important in HCMV replication (41, 81). Importantly, ErbB2 and ErbB4 signaling signatures were enriched among P2Y2-dependent genes during HCMV infection (*SI Appendix*, Fig. S3). The ErbB signaling network is often dysregulated in cancer (82) and plays a role in cell proliferation, differentiation, apoptosis, and migration (82). ErbB4 via PI3K/Akt signaling was shown to promote gastric cell proliferation (83) and support mesenchymal stem cell characteristics (84). P2Y2 was found to activate EGFR and ErbB3 (126); therefore, our results showing P2Y2-dependent activation of Akt in HCMV-infected cells (Fig. 7) stimulate us to speculate about a possibly unappreciated interaction between P2Y2 and ErbB2/ErbB4 in regulation of Akt activity and other downstream processes important for both viral replication and cancer.

In summary, our results show that P2Y2 and P2X5 receptors play critical roles within HCMV-infected cells, and set the stage for additional investigation of the impact of this receptor family in HCMV biology. Furthermore, our observation that a pharmacological block to P2Y2 dramatically reduces viral yield raises the possibility that P2Y2 antagonists, if well tolerated, could prove to be attractive candidates for new HCMV therapies.

Materials and Methods

Detailed methods are provided in *SI Appendix, SI Materials and Methods*. These describe cells used; cloning strategies; viruses and chemical compounds used; RNA-seq analysis; RNA, DNA, and protein analysis; siRNA knockdown procedure; viral entry assay; intracellular calcium assay; wound healing and transwell migration assays; and statistical analysis.

Data Availability. Raw RNA-seq data are available from the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE130665.

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