Differential Disruption of Nucleocytoplasmic Trafficking Pathways by Rhinovirus 2A Proteases

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ABSTRACT The RNA rhinoviruses (RV) encode 2A proteases (2A^{Pro}) that contribute essential polyprotein processing and host cell shutoff functions during infection, including the cleavage of Phe/Gly-containing nucleoporin proteins (Nups) within nuclear pore complexes (NPC). Within the 3 RV species, multiple divergent genotypes encode diverse 2A^{Pro} sequences that act differentially on specific Nups. Since only subsets of Phe/Gly motifs, particularly those within Nup62, Nup98, and Nup153, are recognized by transport receptors (karyopherins) when trafficking large molecular cargos through the NPC, the processing preferences of individual 2A^{Pro} predict RV genotype-specific targeting of NPC pathways and cargos. To test this idea, transformed HeLa cell lines were created with fluorescent cargos (mCherry) for the importin α/β , transportin 1, and transportin 3 import pathways and the Crm1-mediated export pathway. Live-cell imaging of single cells expressing recombinant RV 2A^{Pro} (A16, A45, B04, B14, B52, C02, and C15) showed disruption of each pathway with measurably different efficiencies and reaction rates. The B04 and B52 proteases preferentially targeted Nups in the import pathways, while B04 and C15 proteases were more effective against the export pathway. Virus-type-specific trends were also observed during infection of cells with A16, B04, B14, and B52 viruses or their chimeras, as measured by NF- κ B (p65/Rel) translocation into the nucleus and the rates of virus-associated cytopathic effects. This study provides new tools for evaluating the host cell response to RV infections in real time and suggests that differential 2A^{Pro} activities explain, in part, strain-dependent host responses and diverse RV disease phenotypes.

IMPORTANCE Genetic variation among human rhinovirus types includes unexpected diversity in the genes encoding viral proteases (2A^{Pro}) that help these viruses achieve antihost responses. When the enzyme activities of 7 different 2A^{Pro} were measured comparatively in transformed cells programed with fluorescent reporter systems and by quantitative cell imaging, the cellular substrates, particularly in the nuclear pore complex, used by these proteases were indeed attacked at different rates and with different affinities. The importance of this finding is that it provides a mechanistic explanation for how different types (strains) of rhinoviruses may elicit different cell responses that directly or indirectly lead to distinct disease phenotypes.

KEYWORDS 2A, live-cell imaging, nuclear export, nuclear localization, nuclear trafficking, protease, rhinovirus

The controlled trafficking of protein and RNA across the nuclear membrane is essential for many eukaryotic cellular processes. Nuclear pore complexes (NPC) are large proteinaceous channels embedded in the nuclear envelope that restrict and

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regulate such transport. Each of the estimated 2,800 NPC per HeLa cell is assembled from about 30 different proteins, or nucleoporins (Nups), arranged with 8-fold radial symmetry (1). Small proteins (<40 kDa), ions, and equivalent metabolites are able to freely diffuse through the NPC, but larger macromolecules (cargos) cannot, by themselves, navigate the disordered, hydrophobic Nup tangle that constitutes the central channel. Instead, appropriate directionally targeted cargos display short nuclear localization signals (NLS) or nuclear export signals (NES) that are recognized and bound by karyopherin receptors (importins or exportins) traveling in the required direction. The cargo/receptor selection process is regulated by a RanGTPase-moderated energy gradient, which determines the active or inactive status of the various karyopherins (2). Properly assembled, an import/export complex can readily shuttle through the NPC via transient interactions with Phe/Gly-rich motifs on the Nups lining the central channels of the pores (1).

Viruses with nuclear replication cycles rely on these canonical pathways to import or export their proteins and genomes (3, 4). Cytoplasmic viruses, which do not require the same processes, may instead utilize the NPC as an attractive antihost target. Disruption of active import can restrict large, otherwise nuclear-targeted proteins to the cytoplasm, where they can be usurped for viral RNA and protein synthesis. Trafficking inhibition can also block the import of antiviral transcription factors and the subsequent export of induced antiviral mRNAs that could hinder virus replication (5). The RNA picornaviruses are particularly adept at NPC disruption. For cardioviruses, such as encephalomyocarditis virus (EMCV), the amino-terminal leader protein (L) induces hyperphosphorylation of Phe/Gly nucleoporins, Nup62, Nup153, and Nup214, through a Ran-dependent, mitogen-activated protein kinase cascade (6). In contrast, enteroviruses, like poliovirus and rhinovirus (RV), use a mechanism whereby viral 2A proteases (2A^{Pro}) cleave a subset of Phe/Gly nucleoporins, including Nup62, Nup98, and Nup153 (7–9). Whether by cleavage or phosphorylation, the consequence is an inability of karyopherin-cargo complexes to actively traverse the central channels of the pores. Nuclear proteins (e.g., nucleolin, PTB, Sam68, and La autoantigen) are free to diffuse from their normal locales and accumulate in the cytoplasm, where they become accessible to the virus for internal ribosomal entry site (IRES)-dependent polyprotein translation and genome replication (9). While the EMCV L-dependent phosphorylation process inhibits almost all active transport into and out of the nucleus, the enterovirus 2A^{Pro} pathways are more selective. Poliovirus and rhinovirus infections have subtly different transport inhibition phenotypes, suggesting that their respective 2A^{Pro} have different targeting objectives within the NPC (10).

Indeed, for the rhinoviruses, recombinant 2A^{Pro} from three different species (A, B, and C), or even from different genotypes within these species, select alternative Nup targets or cleave the same targets but at quite different rates when assayed *in vitro* (11). Why do rhinoviruses encode such diversity in an essential enzyme? The *in vitro* experiments imply that selective Nup cleavages could lead to *in vivo* differences in NPC transport or phenotypes and thus would be unique to an initiating virus genotype. In other words, the preferred 2A^{Pro} sequences carried by different rhinoviruses (or other enteroviruses) may help explain the strain-specific phenotypic differences in cellular gene expression. If these viruses allow certain transport pathways to be active while blocking others, the avidity of 2A^{Pro} for unique cohorts of Nups should then correlate with different nuclear transport shutoff phenotypes. We report here a live-cell fluorescent imaging assay measuring nuclear cargo trafficking dependent on specific importin/exportin pathways. Introduction of a panel of RV 2A^{Pro} (A16, A45, B04, B14, B52, C02, and C15) into the system had effects dependent upon the RV genotype. Similar trends were confirmed for a subset of these viruses during cell infections, as monitored by differential effects on the nuclear translocation of the p65(RelA) subunit of the NF- κ B antiviral transcription factor complex. The data support the idea that individual 2A^{Pro} selectively interfere with cellular gene expression, intracellular signaling, and protein localization to elicit different RV phenotypes in their hosts.

FIG 1 Cleavage of NPC Nups by 2A^{pro}. Reaction mixtures containing isolated HeLa cell nuclei (6×10^6) and recombinant 2A^{pro} (0.2 nmol) were incubated at 35°C for 4 h. Aliquots were fractionated by SDS-PAGE and then visualized after Western analyses (MAB414).

RESULTS

2A^{pro} cleavage preferences. A previous panel of recombinant RV 2A^{pro} from RV A16, A89, B04, B14, Cw12 (C02), and Cw24 (C06) showed processing differences among enzymes when assayed against substrates that included eIF4G (HeLa cytoplasmic extracts), Nup62 (recombinant), or nuclear Nups (HeLa nuclei) (11). However, some enzyme pairs did behave similarly (e.g., A16 versus A89), and it was not entirely clear whether the 2A^{pro} activities were differentiated according to the 2A^{pro} genotype clades or more generically among homologous species lineages (A, B, or C). Accordingly, the panel was augmented with 8 new preparations (A01a, A02, A28, A45, A95, B17, B52, and C15) so that the collection then included representatives of all major 2A^{pro} clades as defined for the currently recognized 166 genotypes (11). The closest sequence pairs (>86% amino acid identity; A01a/A02, A02/A16, and B17/B52) and the most divergent sequence pairs (<40% amino acid identity; any B relative to any A or C) bracketed an average 2A^{pro} identity of 55% within the panel.

As before, the full collection showed variation in its preferences for Nup substrates (Fig. 1), primarily Nup62 and Nup153, when monitored with a pan-Nup antibody. The diverse bands indicate different cleavage profiles. For RV-A, the A45 and A95 enzymes from clade D (12) differed significantly from the more common profiles of A01a, A02, A16, and A28. From additional data collected after 1-h and 8-h reactions (not shown), it was clear this particular pair was actually cleaving identical substrate sites, but A45 achieved completion nearly twice as fast as A95. Within RV-C, all three tested enzymes gave similar-size monoclonal antibody (MAb)-reactive bands, but again, the rates at which these bands appeared or disappeared varied. As previously noted, all RV-C 2A^{pro} were particularly adept at Nup62 cleavage, and this band was among the first to disappear (11). The RV-B proteases generated multiple MAb-reactive bands unique to the species and sometimes to their internal clades. For example, all RV-B proteases cleaved near the C terminus of Nup153, but B14 removed only a small fragment, while B52 cleaved at a different site and removed a larger fragment. Each of the RV-B enzymes, though, failed to process Nup62. In total, the assessment of a larger panel of 2A^{pro} highlighted clear differences within and between species and genotypes. The overall diversity in cleavage phenotype clades was reasonably represented by the subset of enzymes including A16, A45, B04, B14, B52, C02, and C15.

Cell line validation. A prediction from the Nup substrate findings is that differential Nup cleavage by the panel of 2A^{pro} should manifest as differential transport pathway disruption in cells. Controlled, synchronized RV infections are difficult, and in any case, the required membrane receptors for RV-C are absent from cultured cells, as the viruses do not infect any natural native monolayers (13). Achieving a sensitive, reproducible measure of comparative 2A^{pro} activities required development of cell and protease fluorescent-labeling techniques. Four transformed HeLa cell lines that constitutively express mCherry reporter proteins were created. Each reporter was linked C terminally

FIG 2 mCherry cell lines. (A) Expressed mCherry fusion proteins linked to NLS or NES are described in Materials and Methods. (B) Transduced HeLa cells correctly localized mCherry signals to the nuclei or cytoplasm or, if there was no fusion fragment (none), to both. DIC, differential inference contrast. (C) Protein localization signals and the transport receptor responsible for shuttling the mCherry fusion proteins. Image capture was at $\times 10$ magnification.

to an NLS or NES segment that directed its cargo trafficking according to a different importin/exportin pathway (Fig. 2A). The pathways with definable localization sequences (Fig. 2C) included importin α/β (e.g., simian virus 40 [SV40] NLS), transportin 1 (e.g., M9 NLS), transportin 3 (e.g., RS NLS), and Crm1 (PKI NES). Cell imaging readily showed the mCherry signals were properly directed by their localization signals (Fig. 2B). The NLS proteins accumulated in nuclei. The NES protein was predominantly cytoplasmic. These modified mCherry proteins are <40 kDa, so if their active trafficking is disrupted, each will quickly diffuse throughout the cell to equilibrium.

Transfection assay validation. Sequence differences among RV 2A^{Pro} preclude the use of a common tracking antibody, which in any case would be ineffective in intact cells, but each enzyme has in common an efficient proteolytic activity directed at its own 1D/2A site, the segment that links it within its polyprotein. Therefore, the relative 2A^{Pro} expression was monitored by configuring the genes in frame to a green fluorescent protein (GFP) reporter sequence, using a sufficient (29- to 33-codon) homologous 1D spacer, so that the translated fusion proteins would efficiently self-process (Fig. 3A). The GFP signal then acts as a surrogate for the amount of active 2A^{Pro}. A Flag tag was added at the C terminus of each 2A^{Pro} to prove that assumption. Translation of the transcript panel in reticulocyte lysates showed similar protein expression levels and confirmed that the encoded enzymes were active for complete self-processing at their respective 1D/2A sites (Fig. 3C). Cellular translation from transfected transcripts gave similar patterns (Fig. 3D). This translation was driven by an EMCV IRES, without which 2A^{Pro} cleavage of eIF4G would inhibit continued transcript expression (11). Interestingly, for the parallel control plasmids, which expressed only GFP or an inactive mutated (GFP-2A_{mut}) protein, cellular expression, even though driven by this IRES, was never as robust (Fig. 3D). 2A^{Pro} inhibition of competitive cap-dependent translation was almost certainly the cause of this (14).

The expressed proteases, particularly from B14 and B52, were also adept in cells, but not reticulocyte lysates, at trimming the homologous 1D segment from the C termini of their GFPs. This did not affect the relative GFP fluorescence because the protein's activity is tolerant of short C-terminal extensions (15). Moreover, in no case did 2A^{Pro} expression compromise mCherry concentrations or remove the linked NPC targeting signals. Equivalent mCherry proteins were present in all the cells, for all NLS/NES lines,

FIG 3 Transcript design and cleavage verification. (A) GFP-containing RNA transcripts. The arrows indicate a 2A^{Pro} self-cleavage site between the viral capsid 1D protein fragment or GFP and 2A itself. (B) Transduced mCherry reporter cells were transfected with tGFP-2A and then lysed 12 h p.t. The integrity of mCherry was verified in Western assays relative to tubulin. The right-hand lanes have unmodified mCherry markers from control cells. (C) tGFP-2A was translated in reticulocyte lysates. Protein detection after SDS-PAGE fractionation was by Western analyses. (D) Proteins expressed in HeLa cells after transfection with tGFP-2A were analyzed similarly to panel C. Cell collection was at 8 h p.t. except for control samples (tGFP and A16*, 16 h p.t.), which required longer incubation for GFP detection. Relative band intensities by densitometry (Norm) were normalized to A16.

and for all 2A enzymes up to at least 12 h posttransfection (p.t.) (Fig. 3B). When these elements were combined, the correlation between mCherry localization and GFP (2A^{Pro}) intensity could be documented. Figure 4 shows examples of static, fixed cell images showing that transfection-introduced transcripts with active (GFP-2A), but not inactive (GFP-2A_{mut}), enzyme redistributed the nuclear mCherry (M9 NLS) signal throughout the cell or, conversely, redistributed a cytoplasmic mCherry (NES) signal into a similar, diffuse pattern equivalent to that achieved by leptomycin B. For both illustrated cell types, and as extended to all four cell lines (data not shown), 2A^{Pro} cleavage of the relevant transportin-required Nups was responsible for this effect.

Live-cell image capture. Fixed cell images do not record changes in cleavage rates that were likely properties of the enzyme panel or compensate for cell expression individualities. Fortunately, the inherent fluorescence of GFP, mCherry, and DAPI (4',6-diamidino-2-phenylindole) does not require fixation for visualization. The transformed cells were plated and grown in a microscope chamber where multiple field positions and wavelengths could be recorded simultaneously with time-lapse imaging. Computational image processing (JEX) allowed the nuclear and cytoplasmic signals for individual cells to be sensitively and independently tracked throughout the course of an experiment. The recordings began 2 h after transfection with the characterized GFP, or GFP-2A^{Pro} transcripts, under conditions equivalent to those described above, which validated the cells and RNAs. The data from any cell that eventually became "green" (i.e., was transfected) was captured. A typical plot from an mCherry M9 NLS recording

FIG 4 2A^{pro} disruption of nuclear transport. mCherry-M9 NLS (A) and mCherry-PKI NES (B) cells were transfected with GFP or A16 GFP-2A pCITE transcripts. At 10 h p.t., the cells were fixed and stained with DAPI or treated with 6 nM leptomycin B for 30 min and then fixed and stained with DAPI (B, right column). Image capture was at $\times 10$ magnification.

(15-min intervals) illustrates one control cell (Fig. 5A), tracking steady-state nuclear and cytoplasmic mCherry signals for up to 12 h. The starting mCherry signal was predominantly nuclear because of the encoded NLS. Introduction of 2A^{pro} (GFP-2A A16) rapidly changed this pattern (Fig. 5B and C). These representative tracks document nuclear mCherry efflux in almost exact proportion to reciprocal accumulation in the cytoplasm over time. Figure 5D records a transfected mCherry-PKI NES cell, with the opposite redistribution pattern after GFP-2A (A16) transfection. Although individual cells varied somewhat in their specific dynamics, especially for GFP expression, there was little cumulative mCherry signal loss over time. This is a stable protein within individual cells, even though the total mCherry signal fluctuated between particular cells. In a strict context, three-dimensional (3D) images are generally required for absolute quantification of nuclear/cytoplasmic localization of protein. However, even the results of this two-dimensional (2D) method demonstrate the sensitive and appropriate behavior of the readout for comparing the different experimental conditions.

Among the variables here are the appearance, rates, and strengths of the GFP signals as surrogates for 2A^{pro} concentrations. Transfected cells may separately take up different amounts of transcript or delay transcript translation according to their indi-

FIG 5 Single-cell fluorescent profiles. Live-cell imaging of transduced HeLa cells transfected with 2A pCITE transcripts was as described in Materials and Methods. The fluorescence intensities for nuclear and cytoplasmic mCherry, nuclear DAPI, and whole-cell GFP were measured in single cells at 15-min intervals for 8 h, beginning 2 h p.t. (A) Example of recorded fluorescent signals in a control nontransfected mCherry-M9 NLS cell. (B and C) Examples of recorded fluorescent signals in A16 2A^{pro}-transfected mCherry-M9 NLS cells. (D) Example of recorded fluorescent signals in an A16 2A^{pro}-transfected mCherry-PKI NES cell.

FIG 6 2A^{pro} transport disruption in single cells. Δ ratioN and Δ ratioC were measured at several time points (3.5, 4, 5, 6, 7, and 8 h p.t.) and normalized to cellular GFP fluorescence intensities in >200 transfected cells for each 2A^{pro} as described in Materials and Methods. The results from one experiment with the mCherry-M9 NLS cells are presented as scatter plots for 4 and 6 h p.t. Each point represents a single cell. The gray points are outliers (the upper and lower 5%), and the black points represent the median 90% of analyzed cells. The mean GFP normalized Δ ratios of the median 90% of cells were calculated and are shown below the graphs. The differences (P values) between the means (t test; PRISM) for each pair of proteases were <0.0001, except as indicated.

vidual cycles. The question asked for each cell, each cell line, and each 2A^{pro} was how much GFP signal was required to achieve an equivalent, measurable trafficking impact. As described in Materials and Methods, for each of 200 to 250 cells per condition, the ratio of nuclear mCherry integrated intensity to the total cellular mCherry integrated intensity (nuclear mCherry ratio [ratioN]) was calculated from the recorded images at every time point throughout a given experiment. In the mCherry-NES cells, where the signal starts predominantly as cytoplasmic, the ratio of the cytoplasmic mCherry integrated intensity to the total cellular mCherry integrated intensity (cytoplasmic mCherry ratio [ratioC]) was calculated instead. The change in ratio (Δ ratioN or Δ ratioC) relative to the starting time point (2 h) was then projected over multiple intervals (3.5, 4, 5, 6, 7, and 8 h p.t.) and normalized to the amount of GFP present in that cell (common filter settings) during the same time interval. The method basically evaluates the observable rate of mCherry nuclear influx/efflux as a function of the GFP concentration (i.e., 2A^{pro}) per cell over time.

Comparative transport disruption by 2A^{pro}. The matched panel of seven GFP-2A transcripts was tested comparatively. Four experimental repetitions for each condition were recorded, tracking 200 individual cells each time. Examples of scatter plots (Fig. 6) for just 2 time intervals from only one such experiment show how some 2A^{pro} varied considerably in their abilities to redistribute the mCherry (M9 NLS) signals. Each point represents 1 cell of the 200 plotted for each condition. The gray points are outliers (the upper and lower 5%), and the black points are the median 90% of analyzed cells for that condition. The mean GFP-normalized Δ ratioN of the median 90% of these cells was calculated and is shown below the graphs. Put simply, the recordings allowed statistically significant mean values to be assigned to the amount of mCherry diffusion (i.e.,

FIG 7 Comparative 2A^{pro} disruption efficiencies. Relative Δ ratioN or Δ ratioC values induced in GFP-2A-transfected cells were calculated from the mean GFP normalized values measured for the median 90% of cells at 3.5, 4, 5, 6, 7, and 8 h p.t. for replicate experiments performed with each mCherry reporter cell line. The highest measured mean Δ ratio in a given experiment was set equal to 1 for calculations of relative values. The relative Δ ratios induced by each 2A^{pro} and plotted for each mCherry reporter and its cognate trafficking pathway are the mean values from 4 replicate experiments (M9 NLS, transportin 1; RS NLS, transportin 3; SV40 NLS, importin α/β) or 3 replicate experiments (PKI NES, Crm1). The error bars indicate standard deviations.

nuclear transport inhibition) induced in cells over time for equivalent amounts of GFP signal (i.e., 2A^{pro}).

The cumulative information for four repetitions summarizing ~ 900 h of recordings that tracked $\sim 50,000$ cells is plotted in Fig. 7. For inhibition of the three importin pathways, B52 and B04 2A^{pro} were always more effective than any other enzymes. As early as 3.5 to 4.0 h p.t., B52 and B04 transcripts induced significantly more mCherry nuclear efflux per unit of GFP (2A^{pro}), and they remained the leading enzymes all the way out to 8 h. In cells where mCherry was regulated by the transportin 1 or importin α/β pathway, B14 and C15 were the next most efficient, releasing labeled cargo from the nuclei almost twice as fast as A16, A45, or C02 2A^{pro}. This pattern did not hold, however, for the transportin 3 pathway, where only B04 and B52 distinguished themselves. Phenotypes for the Crm1 export pathway took much longer to manifest for all of the enzymes. By 6 h p.t., though, the B04 and C15 samples were clearly ahead of the others at inducing nuclear influx. These sensitive measurements also recorded C02 and A45 as slightly faster than the remainder when attacking the same pathway if allowed to proceed for the full 8 h.

When the same Δ ratioN and Δ ratioC values were collected and replotted for each individual enzyme (not shown), there was a clear, repeated preference for the order in which the 4 tested pathways were compromised. Transportin 1, transportin 3, importin α/β , and then Crm1 became sequentially impaired, albeit within time frames that varied among the enzyme panel, as described above. That there were indeed overall differences in the observed kinetics and in the extent of inhibition of each pathway was the result predicted by the individual Nup cleavage assays, since it is known that each

FIG 8 Reporter relocalization during infection. (A) Dual transformed PKI NES and YFP SV40 cells were infected with A16 virus (MOI = 10). The cell fields were imaged every 15 min for 14 h (times are shown in each image). An example of single-cell progression is shown. (B and C) mCherry-p65/RelA cells were infected with A16 virus with image capture as in panel A (B) or treated with TNF- α (10 ng/ml) with image capture at 1-min intervals for 20 min (C).

transport receptor interacts with a different cohort of Nups or at distinct sites within the same Nups as they carry their cargo through the NPC (16–20). The data are entirely consistent with the idea that members of the 2A^{pro} panel exerted their individual proclivities against these Nups with different affinities and turnover rates. The mechanistic consequences manifest as actual, recordable, significant transport differences for these representative pathways.

Comparative transport disruption during infection. The different 2A^{pro} activities also predict measurable consequences with regard to antihost activities, particularly cytokine induction, during virus infections. Accordingly, a fifth HeLa cell line was engineered, stably expressing an mCherry-tagged version of the RelA(p65) subunit of the NF- κ B RelA/p50 antiviral transcription activator complex (21). Treatment of the mCherry-RelA cells with tumor necrosis factor alpha (TNF- α) produced rapid relocalization of cytoplasmic NF- κ B into the nucleus (Fig. 8C). RelA proteins encode both NLS and NES, so as an appropriate control, a sixth cell line was also engineered, transforming the previous mCherry-PKI NES cells with an additional yellow fluorescent protein (YFP)-SV40 NLS fusion sequence. The dual reporters in this line respond independently to export (mCherry) and import (YFP) disturbances. Cells from both new lines were infected with A16, B04, B14, and B52 viruses and monitored by time-lapse microscopy. Since infected cells round up and die much more rapidly than after RNA transfections, data processing precluded the standard analyses with JEX. Instead, the phenotypes were evaluated using per-cell digital quantitation of images captured with the relevant filters (22) and included only those cells that eventually exhibited cytopathic effects (CPE), meaning they were indeed productively infected. Figure 8A to C shows sample images for both cell lines (A16 infection). The data summaries (Fig. 9) followed ~100 cells per sample over 14 h, recording the time of CPE, as well as virus-dependent NPC transport disruption (mCherry and YFP) or NF- κ B disruption (mCherry). Infection of the dual-reporter cells (PKI NES and YFP-SV40 NLS) with B52 or B04 led to equivalent, rapid disruption of active nucleocytoplasmic trafficking. At least half of each reporter signal was relocalized by 6 h postinfection (hpi) with either virus (Fig. 9A and B). The B14-infected cells required more time (8.5 hpi) for the same effect, and A16-infected

FIG 9 Transport disruption during virus infection. (A to D) Dual-transformed PKI NES with YFP SV40-NLS cells were infected with A16, B04, B14, or B52 (A, B, and D) or chimeric B14-based (C) rhinoviruses. Individual cells (100) in captured cell field images (15-min intervals for 14 h) were visually scored for cytoplasmic/nuclear mCherry signals (A), YFP signals (B), or CPE (D) at each time point (see Materials and Methods). The values were averaged ($n = 2$ separate experiments) and plotted. (E and F) Similar to panels A and D, mCherry-p65/ReIA cells were infected with the indicated viruses. The captured images (100 cells per RV infection; $n = 2$ separate experiments) were evaluated for signal relocalization (E) and CPE (F).

cells were by far the slowest to respond (9.25 hpi for NLS and 9.75 hpi for NES). For the B52 and B04 viruses, CPE was half maximal at approximately 3 to 3.5 h post-NPC disruption (9 and 9.5 hpi, respectively) (Fig. 9D), which is 1.5 to 2.5 h earlier than for A16 and B14 (11.5 and 11 hpi, respectively).

Assignment of these phenotypes specifically to the included 2A sequences required parallel testing of matched chimeric viruses. RV-A and RV-B swaps are incompatible because of the competing locations of the *cis*-acting replication elements (within 2A for RV-A and within 1D for RV-B) and a requirement for short homologous cleavage site sequences during the *cis*-acting 2A processing event (23). Nonetheless, two fully viable recombinant B14 viruses were obtained that replaced (or not) the native B14 2A gene with the 2A gene from B52 in identical contexts that repeated (or duplicated) 8 codons at each end of the gene. When the B14/B52 2A virus infected the dual-reporter cell line, it disrupted YFP-SV40-NLS import (Fig. 9C) faster than the control chimeric B14/B14 2A virus, with the inflection points occurring at 7.5 hpi and 9.25 hpi, respectively. The export inhibition of mCherry PKI NES, measured in parallel, virtually overlapped the respective YFP signals, and for both chimeras, CPE occurred approximately 2.5 to 3 h after the midpoint of trafficking inhibition (data not shown). Basically, the time differ-

ential between the chimeras was similar to that of the native B52 and B14 viruses (Fig. 9A), confirming that transport disruption rates were conferred by the 2A^{pro} sequences.

Similar trends were also observed in mCherry-RelA-infected cells (Fig. 9E). This cytokine-inducing transcription factor became nuclear at least 1 h earlier during B04 and B52 infections than with B14 or A16, but the timing of these events was delayed from 30 min (B14) to 2.5 h (B04, B52) relative to the YFP SV40 NLS cargo (Fig. 9B) or the PKI NES cargo (Fig. 9A). Interestingly though, A16 infection induced mCherry-RelA translocation almost an hour earlier (8.5 hpi) than its inhibition of nucleocytoplasmic transport in the PKI NES and YFP-SV40 NLS cells. Moreover, the activation of this antiviral pathway (overexpression of the recombinant gene) seemed to directly influence the parallel observation of CPE, which was delayed relatively during B04, B14, and B52 infections while it was accelerated (to 10.5 hpi) in the A16 infections (compare Fig. 9D and F). Thus, each virus caused markedly different effects on specific cargoes undergoing regulated nucleocytoplasmic trafficking. In particular, the differing effects on mCherry-RelA trafficking are consistent with the idea that each virus programs a unique host response, potentially regulated by its overall replication rate and its preferred specificity for Nup cleavages.

DISCUSSION

2A^{pro} is unique to the genus *Enterovirus*. During infection, all 2A^{pro}, regardless of species or isolate, participate in primary polyprotein processing. Thereafter, they target initiation factor eIF4G to achieve translation inhibition of capped cellular mRNAs. The 2A^{pro} then progress to Nup protein cleavages to bring about an inhibition of nucleocytoplasmic trafficking (7, 9, 24). However, virus isolates, particularly between the RV species, are not universal in their 2A^{pro} sequences, especially within the substrate binding pocket (25). These changes cause site selection preferences within eIF4G substrates and within specified sets of target Nups, as measured *in vivo* or *in vitro* (9, 11, 26–29). This range of activities was examined here with a panel of 13 RV enzymes chosen to cover the clade diversity within the RV 2A^{pro} phylogeny. As reported previously, but now confirmed with a larger panel, the substrate preferences manifest as rate or location differences within the Nups, but the variation was finite in that some clades of enzymes had similar proclivities. A01a, A02, A28, and A16 formed one grouping, and A45 and A95 (clade D viruses) formed another, as did B17 and B52 and also C02 and C06.

Rhinoviruses, the most prevalent of human respiratory viruses, are responsible for at least 50% of recorded upper respiratory tract illnesses (30). RV infections also cause a spectrum of etiologies, from asymptomatic to pneumonia or severe asthma exacerbations (31). The genotypes of individual RVs influence illness severity (32). Recent data from the University of Wisconsin (UW) COAST (Childhood Origins of Asthma) study associated certain RV-A and RV-C isolates with more severe respiratory illnesses in infants than the equivalent RV-B isolates (32). Moreover, sinus epithelial cells differentiated at the air-liquid interface, tested with recombinant A16, A36, B52, B72, C2, C15, or C41 viruses, also showed that the RV-B types have correspondingly slower replication and lower cytotoxicity and cytokine/chemokine production than the RV-A and RV-C viruses (33). Since the RVs infect only a small subset of airway epithelial cells, virus-induced lysis cannot be the singular cause of observed damage to the epithelial cell lining (34). Rather, differential immune responses triggered by these infections contribute significantly to respiratory symptoms (35). The infected and bystander airway epithelial cells produce a number of proinflammatory cytokines and chemokines (e.g., interleukin 8 [IL-8], IP-10, granulocyte colony-stimulating factor [G-CSF], and RANTES) that correlate with RV symptom severity (36–38). The generation of these responses requires a network of intracellular and extracellular immune signaling dependent upon nucleocytoplasmic trafficking of proteins and RNA.

We hypothesized that genetic diversity among 2A^{pro}, which clearly manifests as cleavage differences in cytokine-trafficking pathways, could be a determinant in the disease expression of individual RV genotypes. If true, the observed Nup cleavage

differences should show differential inhibition of nuclear transport pathways through NPC, providing each virus group a mechanism of control over the types of nuclear trafficking. Initially, 4 HeLa cell lines stably expressing mCherry marker proteins linked to NLS or NES sequences for importin α/β , transportin 1, or transportin 3 import or Crm1 export were transfected with RNAs encoding 7 representative 2A^{pro} in a live-cell fluorescence assay. The inherent activity of 2A^{pro} inhibits self-expression from cDNA-derived capped mRNAs. Therefore, to control for differential transfection and protein synthesis levels per cell, the RNA transcripts included a linked GFP gene driven by an EMCV IRES as a surrogate concentration signal. Each 2A^{pro} activated itself by N-terminal autoprocessing as it would within its own polyprotein. An advantage of this assay was the ability to record cell-to-cell variability over time, thus sampling the full population heterogeneity and breadth of responses.

As predicted by the Nup cleavage hypothesis, on a per-enzyme basis, the 2A^{pro} targeted these pathways with different avidities. The B52 and B04 proteases disrupted the importin α/β , transportin 1, and transportin 3 nuclear import pathways more efficiently than other proteases, and for each pathway, there was a shifting hierarchy of rates across the panel, varying reproducibly 2- to 10-fold over 8 h, depending upon the 2A^{pro}. For the Crm1 export pathway, the C15 and B04 proteases were the most efficient, and again, the relative rates were dependent on the selection of 2A^{pro}, with the highest (C15) exceeding the lowest (B52) by nearly 4-fold. Interestingly, in reported nonquantitative immunofluorescence (IF) assays with poliovirus 2A^{pro}, it was suggested that this particular enzyme targeted importin α/β and transportin 1 import pathways, but not Crm1 export (9, 39). Our data found all RV enzymes readily impacted the export pathway, albeit some did it more slowly and with a lower apparent priority. In this regard, perhaps poliovirus simply has rate preferences more similar to the B52 pattern.

In general, the RV-A and RV-C proteases showed slower kinetics than B04 and B52 in disrupting nuclear import. Were this to translate into patient infections, it is conceivable that some antiviral signaling might then occur with these viruses before full nuclear transport shutoff, thus promoting proinflammatory immune responses that could cause the more severe illness symptoms observed with RV-A and RV-C infections (33). Indeed, the nuclear cycling of NF- κ B, when measured in a new reporter cell line, was dependent upon the type of RV that infected the cells (Fig. 7C), as was the time of appearance of CPE (Fig. 7B and D). While infections measure the collective effects of 2A^{pro} damage during an infectious cycle, the data are entirely consistent with the per-enzyme rate kinetics observed in the RNA transfection assays. The nuclear import pathways studied here are also responsible for the import of a number of nuclear proteins (transportin 1, hnRNP A1, transportin 3, Srp20, importin α/β nucleolin, and PTB) that relocate into the cytoplasm of poliovirus- and RV-infected cells and can stimulate viral replication and translation (8, 10, 40–45). Timed 2A^{pro} disruption of these pathways could certainly affect overall viral replication levels, which contribute directly to RV virulence. Notably, among the 77 RV-A, RV-B, and RV-C genotypes surveyed in the UW COAST study (32, 46–48), B52 was the least virulent and C02 was the most virulent. The other genotypes in our 2A^{pro} panel were not captured by that study. In our experiments, the B52 protease proved to be one of the most efficient proteases at disrupting the three nuclear import paths and the slowest at regulating the export pathway. The import pathways regulate cargos involved in the expression of immune responses. Transportin 1 and transportin 3 import hnRNPs and SR proteins, respectively, both of which are factors controlling alternative splicing mechanisms of pre-mRNAs (41, 49, 50). Most genes of the immune system, including cytokines, intracellular signaling molecules, and transcription factors, are alternatively spliced by proteins imported via these pathways to either enhance or repress immune responses (51–53). The importin α/β heterodimer imports transcription factors NF- κ B, IRF-3, and STAT1 into the nucleus, where they stimulate the interferon response and upregulate antiviral genes (54–56). Rapid disruption of this nuclear import pathway (e.g., by B52 2A^{pro}) could quickly dampen this type of immune response and reduce the cellular damage and respiratory symptoms caused by proinflammatory cytokines and chemokines.

MATERIALS AND METHODS

Recombinant 2A^{Pro}. Matched bacterial plasmids for the expression of recombinant rhinovirus 2A^{Pro} (genotypes A16, A89, B04, B14, C02, and C06) have been described previously (11). Additional sequences from RV A01a, A02, A28, A45, A95, B17, B52, and C15 were engineered from viral cDNA or RNA templates by cloning the equivalent 1D (a partial COOH fragment of VP1 capsid protein)-2A amplicons into pET11A vectors. Plasmid transformation [BL21(DE3) LysS cells], protein induction, purification by sequential anion exchange, and gel filtration chromatography were as described previously (11). Most of the new proteases (A01a, A02, A28, A45, A95, B04, and C15) were soluble following cell lysis, but B17 and B52 preparations (like B14) required solubilization and dialysis before purification (11). In parallel, amplicons from a subset of the panel [1D(partial)-2A for A16, A45, B04, B14, B52, C02 and C15] were transferred into pCITE 4a (Novagen) vectors that had been engineered to encode N-terminal GFP expression (amplified from pAcGFP1-NUC [Clontech]) from an EMCV IRES and were linked to a C-terminal Flag tag (DYKDDD DK). Transcription of the resulting pGFP-2A plasmids via the vectors' T7 promoters produced cap-independent mRNA transcripts encoding in-frame GFP-1D(partial)-2A-Flag fusion proteins for the respective proteases (tGFP-2A). The A16_{mut} (A16*) sequence was the same as that of A16 except for a 2A^{Pro} substitution, Cys₁₀₆Ala, inactivating the protease (11).

Nup cleavage assay. Purified recombinant 2A^{Pro} (0.25 nM) were reacted with HeLa cell nuclei, similar to previous reactions (11). Basically, cells pelleted from suspension cultures were lysed (10 mM Tris, pH 8.5, 140 mM NaCl, 1.5 mM MgCl₂, and 0.5% IGEPAL at 4°C) (NIB), and then, after collection (500 × g for 5 min at 4°C), the nuclei were washed (2 times, NIB, and then 3 times, TB [20 mM HEPES, pH 7.3, 110 mM KOAc {potassium acetate}, 2 mM MgOAc {magnesium acetate}, 1 mM EGTA]), resuspended, and incubated with enzyme (6 × 10⁶ nuclei in 300 μl of TB; 34°C; 8 h). As required, aliquots were removed, sonicated, combined with gel loading buffer, and then boiled to stop the reactions. Nup cleavage was visualized after SDS-PAGE fractionation and Western analysis using MAb414 (Covance).

GFP-2A cleavage assay. pGFP-2A plasmids (5 μg) were linearized with PmeI and purified (Qiagen PCR cleanup kits) before transcription using T7 RiboMax large-scale RNA production systems (25 μl; 4 h; 37°C; Promega). Following RQ1 DNase treatment (20 min), RNA was extracted with TRIzol (Life Technologies) and then purified according to the manufacturer's protocols. Transcript translation in rabbit reticulocyte lysates (RRL) (Promega) was used to verify endogenous 2A^{Pro} activity and processing from GFP-1D(partial). The products were analyzed by SDS-PAGE and Western analysis using antibodies against GFP (rabbit polyclonal SC-8334; Santa Cruz Biotech) or the 2A C-terminal Flag tag (rabbit polyclonal ab1162; Abcam).

Transformed HeLa cell lines. Cell lines (ATCC CRL-1958) carrying mCherry reporter genes linked to defined NLS/NES sequences were previously reported *in brevis* (39). To summarize, the SV40 large T antigen NLS, the M9 NLS (49), the R5 NLS from splicing factor 2, or the leucine-rich NES from PKI (57) was engineered in frame C terminal to an mCherry gene in the context of an MIGR1-based IRES-neo retroviral vector (NG) that expresses neomycin resistance (58, 59). Vector generation required 293T cell transfection with pNG-mCherry reporter plasmids (4 μg), pMDGag-Pol (4 μg; packaging plasmids), and vesicular stomatitis virus G protein (VSV-G) envelope plasmids (2 μg in 500 μl of Opti-MEM with 20 μl of polyethylenimine). The transfection medium was replaced 12 h p.t. and subsequently harvested and filtered (0.45 μm; 48 h p.t.) by previously described techniques (60). After infection of HeLa cells by spinoculation (61), genome integration, and antibiotic selection, individual colonies of transformed HeLa cells were selected and then expanded to generate stable cell lines. Cells expressing the mCherry-tagged NF-κB subunit, RelA/p65, were derived similarly but used an IRES-puro retroviral vector encoding resistance to puromycin (58). The RelA/p65 gene was a gift from Shigeki Miyamoto (21). A separate, dual YFP NLS and mCherry-NES stable HeLa line was created by retransforming the mCherry-PKI NES line with an IRES-puro retroviral vector carrying an enhanced YFP gene (22) linked to the SV40 NLS. All cell lines were maintained in suspension culture (37°C; Eagle's medium, 10% newborn calf serum (NBCS), 2% fetal bovine serum [FBS] under 5% CO₂).

Viruses. Recombinant RV A16, B14, and B52 and derivative chimeric 2A viruses were produced by transfecting HeLa cells with T7 transcripts synthesized *in vitro* from the respective linearized cDNAs. To ensure proper primary and secondary polypeptide cleavage, the chimeric viruses B14/B14 2A and B14/B52 2A were engineered to replace the precise native B14 2A gene with inserted sequences repeating the 3'- and 5'-terminal 8 codons of capsid proteins 1D and 2A flanking a full, new 2A gene derived from B14 or B52. All resultant infectious progeny, along with RV B04 (ATCC), were amplified in HeLa cells. After CPE was observed, infected cells were supplemented with HEPES (to 50 mM, pH 7.2) and then lysed by multiple freeze/thaw cycles (3 times). Virus from clarified supernatants was pelleted (through 30% sucrose at 140,000 × g at 4°C) before resuspension (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA), and titers were determined by plaque assay.

GFP-2A live-cell image capture. Plated HeLa cells (Eagle's medium, 10% NBCS; 24-well dishes; 37°C) were grown to 60 to 80% confluence. Four hours before transfection (2 μg of tGFP-2A in 250 μl of Opti-MEM Lipofectamine 2000; Invitrogen), DAPI stain was added (4 μg/ml). One hour p.t., the cells were washed (PBS) and the Opti-MEM was replaced with modified Eagle's medium (500 μl with 10% NBCS). The cells were visualized with a Nikon Eclipse Ti80 microscope equipped with an incubation chamber (34°C; 5% CO₂). NIS Elements Ar software captured images every 15 to 20 min for 8 to 10 h, recording 3 channels with 460-nm (blue), 535-nm (green), and 632-nm (red) filters. Tracking of single cells and quantitation of their color images were done with JEX v0.0.4[1], a customized Java-based image capture and batch-processing platform that leverages capabilities provided by ImageJ/FIJI (62; <http://imagej.nih.gov/ij/>, <http://imagej.net>, <http://scif.io/>). The technique recorded the simultaneous nuclear and

cytoplasmic signals for DAPI, mCherry, and GFP (a surrogate for 2A^{pro}) for large numbers of individual cells per condition over the course of each experiment.

Data normalization. Image-filtering and thresholding steps were applied to DAPI (blue) and mCherry (red) images to generate the masks for nuclear, cytoplasmic, and whole-cell areas. Using these masks, integrated intensities (3 wavelengths) were measured for all regions for each cell (600 to 800 cells/set). The values were imported into R software (<http://www.r-project.org>) for filtering and analysis. The integrated nuclear DAPI (blue), integrated nuclear mCherry (red), integrated cytoplasmic mCherry (red), and cellular GFP (green) intensities (in arbitrary relative fluorescence units [RFU]) were plotted over time for each cell within each experimental set. Cells with no changes in mCherry or GFP (i.e., not transfected) were discarded. However, as the background GFP intensity was generally low, the filtering criteria were set loosely to avoid falsely omitting cells that might be expressing some 2A^{pro}. The time tracks were cut short if the cell started exhibiting CPE (>10 h posttransfection). Since the total integrated mCherry intensity of each cell (the sum of nuclear and cytoplasmic integrated mCherry intensities) did not change measurably throughout an experiment (Fig. 5), for mCherry-NLS cells, the integrated nuclear mCherry intensities were normalized by the total integrated mCherry intensities (ratioN) at each time point. Likewise, for the mCherry-NES cells, at each time point, the integrated cytoplasmic mCherry intensities were normalized by the total integrated mCherry intensity (ratioC). The change in the nuclear or cytoplasmic mCherry ratio (Δ ratioN or Δ ratioC) throughout the course of the experiment with respect to the initial ratio was calculated for each 2A^{pro} (200 to 250 cells/2A^{pro} type). The Δ ratioN or Δ ratioC was normalized for cellular 2A^{pro} expression levels by dividing the calculated Δ ratioN or Δ ratioC by the average cellular GFP intensity to compare efficiencies of different 2A^{pro} to disrupt nuclear transport.

Infected live-cell image capture. mCherry (NES; p65/RelA) and YFP (NLS) were monitored during virus infection in wells of plated HeLa cells (as described above) exposed to A16, B04, B14, B52, or chimeric viruses (25°C; 30 min; multiplicity of infection [MOI], 10). The cells were washed with PBS to remove unattached virus prior to incubation and timed image capture (34°C, as described above). Infected cells round and die more rapidly than after RNA transfections. Therefore, data processing precluded standard analyses using JEX, and instead, these phenotypes were evaluated using a per-cell digital analysis (22) where the ratios of cytoplasmic (cyto) to nuclear (nuc) signals were scored visually at standard time points (every 15 min for 14 h) using the following 3-point scale: 0 for a cyto/nuc ratio of >1 (mCherry) or a nuc/cyto ratio of >1 (YFP); 1 for a cyto/nuc ratio of $\leq$ 1 (mCherry) or for a nuc/cyto ratio of $\leq$ 1 (YFP); and 2 for loss of cell motility and/or DNA condensation, consistent with cell death and lysis (i.e., CPE).

Western analyses. Cell monolayers were washed (PBS) and then collected in gel loading buffer (SDS) and boiled. After SDS-PAGE fractionation, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The membranes were blocked with Tris-buffered saline–Tween 20 (TBST) (20 mM Tris, pH 7.6, 140 mM NaCl, 0.5% Tween 20, 10% nonfat dry milk) and then washed (3 times) with TBST before incubation with appropriate primary antibodies (in TBST with 1% nonfat dry milk) overnight at 4°C. The membranes were washed again (3 times) with TBST before incubation with secondary antibodies (in TBST with 1% nonfat dry milk). After final washes, the membranes were exposed to film or imaged on a Foto/Analyst Luminary FX (Fotodyne) in the presence of enhanced chemiluminescence substrate (Promega). Primary antibodies against GFP (rabbit polyclonal SC-8334; Santa Cruz Biotech), mCherry (mouse monoclonal IC51; Abcam), Flag tag (rabbit polyclonal Ab1162; Abcam), α -tubulin (mouse monoclonal T9026; Sigma), MAb414 (Covance), and Nup50 (Ab4005; Abcam) were commercial, as were horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG [A2554; Sigma] and anti-goat IgG [A5420; Sigma]) and anti-rabbit IgG (W401; Promega).

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