Differential Processing of Nuclear Pore Complex Proteins by Rhinovirus 2A Proteases from Different Species and Serotypes[▽]

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Human rhinoviruses (HRVs) from the HRV-A, HRV-B, and HRV-C species use encoded proteases, 2A^{pro} and 3C^{pro}, to process their polypeptides and shut off host cell activities detrimental to virus replication. Reactions attributed to 2A^{pro} include cleavage of eIF4G-I and -II to inhibit cellular mRNA translation and cleavage of select nucleoporin proteins (Nups) within nuclear pore complexes (NPCs) to disrupt karyopherin-dependent nuclear-cytoplasmic transport and signaling. Sequence diversity among 2A^{pro} proteases from different HRV clades, even within species, suggested individual viruses might carry out these processes with unique mechanistic signatures. Six different recombinant 2A^{pro} proteases (A16, A89, B04, B14, Cw12, and Cw24) were compared for their relative substrate preferences and cleavage kinetics using eIF4G from cellular extracts and Nups presented in native (NPC) or recombinant formats. The enzyme panel attacked these substrates with different rates or processing profiles, mimicking the preferences observed during natural infection (A16 and B14). For eIF4G, all 2A^{pro} proteases cleaved at similar sites, but the comparative rates were species specific (HRV-A > HRV-C >> HRV-B). For Nup substrates, 5 of the 6 enzymes had unique product profiles (order of Nup selection) or reacted at different sites within Nup62, Nup98, and Nup153. Only A16 and A89 behaved similarly in most assays. Since each type of karyopherin receptor prefers particular Nups or uses a limited cohort of binding motifs within those Nups, the consequences of individual 2A^{pro} avidities could profoundly affect relative viral replication levels, intracellular signaling, or extracellular signaling, all of which are underlying triggers for different host immune responses.

Human rhinoviruses (HRVs), the major etiological agents of the common cold, are a widespread group of single-stranded, positive-sense RNA enteroviruses in the family *Picornaviridae*. More than 150 different HRV types are recognized, and they are classified into three species, HRV-A, HRV-B, and HRV-C, according to commonalities among their sequences (27). Virus strains within an HRV type generally share at least 87 to 88% amino acid identity within capsid protein VP1. Viruses within the same HRV species share at least 70% identity in the same fragment (36). The genomes of all HRVs, like those of poliovirus (PV) and other enteroviruses, encode a single polypeptide that is co- and posttranslationally processed by virus-encoded proteases, 2A^{pro} and 3C^{pro} (34). Both proteases are additionally involved in multiple host cell shutoff activities that help the virus evade host defense mechanisms and promote its replication. Protease 3C, for example, and/or its precursors cleave several nuclear transcription factors, preventing cellular RNA synthesis by pol1, pol2, and pol3 (8, 9, 43, 44). Protease 2A cleaves translation initiation factors eIF4G-I and -II, inhibiting cellular cap-dependent mRNA translation (15, 19). Additionally, 2A^{pro} targets multiple sites within 4 or more different Phe/Gly-containing nucleoporin proteins (FG Nups) lining the central channel of nuclear pore complexes (NPCs) to disrupt nucleocytoplasmic transport (7, 29). The proteolysis of Nups in cells transfected with a 2A^{pro} cDNA or infected with PV or an HRV correlates temporally with a potent blockage of nuclear trafficking. Simultaneously, many

normally nuclear proteins are observed to efflux into the cytoplasm, accumulating there in unnatural resource pools (e.g., La autoantigen, Sam 68, PTB, and nucleolin) used by the virus to enhance viral internal ribosomal entry site (IRES)-dependent protein translation and genome RNA synthesis (16).

The life cycle of an HRV and other enteroviruses is primarily cytoplasmic. It can be recapitulated efficiently in an Eppendorf tube programmed only with cytosol (26). For cells, however, the movement of proteins and RNA in/out of the nucleus through the NPC is at the requisite core of all gene activation schemes, including those required for nearly every innate immunity trigger. A cell must send appropriate signals (e.g., IRF3, STAT1, or NF- κ B) into the nucleus and transcribe and then export the relevant mRNAs to produce the desired antiviral products. Vertebrate NPCs have multiple copies (8-fold symmetry) of approximately 30 different Nup proteins contributing to structure and transport activities (35). Small molecules (<30 kDa) can passively diffuse in either direction through any NPC, but the movement of larger molecules is active and requires obligate signal-dependent cargo binding to karyopherin transport receptors for sequential slippery-shuttling interactions with FG domains on specific subsets of Nups. The cargo-carrying karyopherins thus wiggle their way (in or out) through the Nup protein tangle within the core channel of the NPC. Each type of transport receptor prefers particular Nups or uses only certain types of FG motifs (35, 39).

The HRV 2A protease that attacks the integrity and permeability of the NPC is a small (~16-kDa) chymotrypsin-like cysteine protease. The full structure of the HRV A02 enzyme has been resolved to atomic resolution (32). A 6-stranded β -core and a 4-stranded β -sheet support a catalytic triad

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(His18, Asp35, and Cys106) similar to that of serine proteases. These residues are oriented within a substrate binding groove made up in part of a β -hairpin loop, the di-tyrosine flap (Ile80-Ile90), which helps the protein accommodate its targets, providing specificity that may extend as far as the P7 to P2' substrate positions (37). The back side of the protein chelates a structurally required zinc ion (32). Much of what is known about the biochemical profiles of HRV 2A^{pro} comes from *in vitro* studies with the protease from A02 or from B14 (37, 38, 42). Given the important jobs carried out by this enzyme on behalf of the virus, it was somewhat surprising when the recent completion of the full set of HRV-A and HRV-B genome sequences (27) highlighted an unexpected degree of variability among the 2A genes from different strains and species. Some pairwise 2A^{pro} amino acid identities range as low as 33% (28), equivalent to the least conserved immunogenic regions of the capsid proteins. The variability extends throughout the mapped substrate recognition groove, including the di-tyrosine flap, as well as to structural support locations, clearly predicting nonequivalent enzymatic properties. Indeed limited assays comparing the B14 and A02 enzymes had already suggested somewhat different specificities (37, 38, 42). Since the HRV-C species are only recently cultured and remain difficult to grow, their 2A^{pro} activities against the NPC or other substrates have never been documented.

We now report the purification and characterization of six recombinant HRV 2A^{pro} proteases (A16, A89, B04, B14, Cw12, and Cw24) selected from dominant clades in all three HRV species. As predicted from sequence differences, the relative 2A^{pro} specificities and individual kinetics against NPC proteins (Nup62, Nup98, and Nup153) and eIF4G varied greatly. The recombinant enzymes, in reactions with isolated nuclei, eIF4G, or recombinant Nup62, had different rates and unique processing patterns, albeit paralleling *in vitro* what was observed during infection with parent viruses. The data support the idea that substantially different HRV phenotypes may be triggered at their core by the capacities of individual 2A^{pro} proteases to selectively interfere with host gene expression, protein localization, and intracellular signaling processes.

MATERIALS AND METHODS

Protein alignments. Full-length polyprotein sequences for HRV-A and HRV-B have been published, as have structure-based sequence alignments (28). MEGA4 (40) was used to compute pairwise sequence distances according to this alignment.

Viruses and cells. Current nomenclature conventions for the HRVs designate the species letter (A, B, or C) and strain genotype (36). Viruses A16 and B14 were derived from infectious recombinant cDNAs (21, 22). ATCC stock viruses B04 and A89 (27), as well as HRV-C patient isolates Cw12 and Cw24, were a generous gift from Wai-Ming Lee (University of Wisconsin—Madison). These particular HRV-C species are only partially sequenced (20), but the available data indicate assignment to the C02 and C06 genotypes, respectively, for which full-length sequences are available (GenBank accession no. EF077280 and EF582387).

HeLa cells (ATCC CRL-1958) were grown in suspension culture (37°C; Eagle's medium and 10% calf serum under 5% CO₂) and then plated 24 h before infection with A16 or B14 (multiplicity of infection [MOI] = 30). After an absorption period (30 min; 20°C; phosphate-buffered saline [PBS]), the cells were washed, overlaid with medium (modified Eagle's medium, 40 mM MgCl₂, 0.1% bovine serum albumin [BSA]), and then incubated (35°C; 5% CO₂). As required by the experiment (typically Western analyses), the plated cells were washed in PBS, collected into gel loading buffer (SDS), sonicated, and then fractionated by Laemmli-SDS-PAGE.

Recombinant HRV 2A^{pro}. A panel of 6 bacterial expression plasmids encoding select HRV 2A^{pro} proteases was constructed. Similar procedures have been described previously (24, 42). Briefly, amplicons encoding the C-terminal portion of the respective VP1 genes (25 to 28 codons) linked to full-length 2A genes were obtained by PCR and templated by cDNA (A16 and B14) or viral RNA (A89, B04, Cw12, and Cw24). After restriction enzyme trimming, the fragments were ligated into pET-11a (Novagen) using NdeI and BamHI restriction sites. An additional A16 derivative (pA16-2A_{C106A}) was constructed by 2-step PCR (with mutagenic primers GGTGATGCTGGTGGGAAATTATTATGC and TCCCA CCAGCATCACCTGCTTCACA). Within this plasmid, the active-site nucleophile codon (C106) was converted to Ala. This construct did not include any VP1 gene sequences. *Escherichia coli* BL21(DE3) LysS cells were transformed with each plasmid and then induced with IPTG (isopropyl- β -D-thiogalactopyranoside). The expressed 2A^{pro} cleaved spontaneously from the VP1-2A fusions. The A16-2A_{C106A} was expressed with an N-terminal Met. After the cells were collected, washed, and sonicated (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF], 5% glycerol), the majority of 2A^{pro} from A16, A89, B04, Cw12, and Cw24 was in the soluble fraction (20,000 $\times$ g for 45 min at 4°C). The B14 enzyme was insoluble. For soluble enzymes (including A16-2A_{C106A}), saturated (NH₄)₂SO₄ (50 mM Tris, pH 8.0) was added to 20% (wt/vol). After incubation (for 12 h at 4°C), the samples were clarified (20,000 $\times$ g for 30 min at 4°C) and then brought to 40% (wt/vol) (NH₄)₂SO₄. Precipitated material (4 to 6 h at 4°C) was collected by centrifugation (15,000 $\times$ g for 30 min at 4°C), resuspended in buffer A (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT), and then fractionated on an ion-exchange column (Bio-Scale Mini Unosphere Q column [Bio-Rad]) pre-equilibrated with buffer A. Elution was with a linear gradient of NaCl (0.05 to 1.0 M in buffer A). The 2A^{pro} fractions (identified by SDS-PAGE) were loaded onto a size exclusion column (HiPrep 16/60 Sepharacyl S-100 HR column; GE Healthcare) pre-equilibrated with buffer A. Gel filtration fractions were analyzed by SDS-PAGE, and those containing 2A at greater than 95% purity (estimated after Coomassie staining) were pooled, concentrated (Amicon Ultra Centrifugal Filters; Millipore), and then dialyzed overnight (buffer A with 150 mM NaCl; 4°C). The B14 2A material was solubilized and refolded before purification. The collected, induced cell pellet was washed (with 1 M NaCl, then 1 M urea, and then H₂O) and solubilized overnight (7 M urea, 50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM DTT, and 5% glycerol at 4°C). After clarification (20,000 $\times$ g for 45 min at 4°C), the supernatant was diluted in the same buffer (to 0.15 mg/ml) and dialyzed (2 times against 50 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mM ZnCl₂, and 5% glycerol) for 12 h and then again (buffer A) for 12 h at 4°C. The (refolded) enzyme was isolated by sequential anion exchange and gel filtration chromatography as described above. Protein concentrations for all preparations were assayed relative to standards (Quick Start Bradford Protein Assay Kit; Bio-Rad).

Recombinant GST-Nup62. The coding sequence for Nup62 was amplified by PCR from pcDNA3.1/HisB-Nup62, a plasmid generously supplied by Nabeel Yaseen (Northwestern University), and then ligated into pGEX-6P2 between the NotI and SalI restriction sites. The construct fuses the Nup62 gene with an N-terminal glutathione S-transferase (GST) tag. *E. coli* BL21 was transformed and then induced with IPTG. The cells were collected, lysed, and sonicated (in 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 mM pepstatin A, and 5% glycerol). Most of the expressed protein was insoluble and collected as a pellet (20,000 $\times$ g for 45 min at 4°C). The material was washed with 1 M NaCl and then 1 M urea, followed by water, before resuspension in lysis buffer containing 6 M urea (4 h at 4°C). After clarification (20,000 $\times$ g for 45 min at 4°C), the supernatant was dialyzed against 2 buffer exchanges (50 mM Tris, pH 7.6, 150 mM NaCl, and 5% glycerol for 12 h at 4°C). Soluble protein was collected with GSTrap (GE Healthcare), eluted (50 mM Tris, pH 8.0, 10 mM reduced glutathione), and then dialyzed (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM DTT) before the protein concentration (Bradford kit; Bio-Rad) and purity (SDS-PAGE and Coomassie) were determined.

2A^{pro} activity with eIF4G. HeLa cells swollen in hypotonic lysis solution (0.75 Mg(OAc)₂, 0.15 mM EDTA, 1 mM PMSF, 0.01 mg/ml leupeptin, 20 mM pepstatin A, 3 mM DTT) were lysed by Dounce homogenization. After clarification (16,000 $\times$ g for 20 min at 4°C), 1/10 volume of 10 $\times$ transport buffer (TB) (20 mM HEPES, pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)₂, 1 mM EGTA) was added before the extracts were snap-frozen for storage (-80°C). Cleavage assays (80 μ l) contained these extracts (40 μ l) and recombinant 2A^{pro} (0.2 μ M) in TB. Samples were incubated (35°C) for up to 2 h, combined with gel loading buffer (SDS), and then boiled to stop the reactions.

2A^{pro} activity with GST-Nup62. Recombinant GST-Nup62 (1 nmol) was reacted with recombinant 2A^{pro} (0.2 nmol) in TB (typically, 200 μ l at 35°C). Aliquots were removed as required, combined with gel loading buffer (SDS), and then boiled to stop the reactions. The products were fractionated by SDS-PAGE

and visualized by Coomassie staining. Protein band intensities were quantitated by densitometry (Total Lab 100 software; Nonlinear Dynamics Ltd.).

2A^{pro} activity with whole nuclei. HeLa cells pelleted from suspension cultures ($1,500 \times g$ for 5 min) were lysed in nuclear isolation buffer (NIB) (10 mM Tris, pH 8.5, 140 mM NaCl, 1.5 mM MgCl₂, and 0.5% Igepal at 4°C). After collection ($500 \times g$ for 5 min at 4°C), the nuclei were washed ($2 \times$ NIB, $3 \times$ TB) and then resuspended in TB (3×10^7 to 4×10^7 nuclei/ml). Nup cleavage assays (300 ml at 35°C) contained 6×10^6 nuclei and 2A^{pro} (1 μ M) in TB with continuous gentle mixing (end over end). Aliquots were removed as needed, sonicated, combined with gel loading buffer, and then boiled to stop the reactions.

Antibody detection. Polyclonal (antibody [Ab]) rabbit serum raised to A16 2A^{pro} has been described previously (1). Primary reagents to eIF4G (rabbit Ab SC-11373; Santa Cruz Biotech), FG-containing Nups (murine MAb 414; Covance), Nup62 (murine monoclonal Ab [MAb] 610498; BD Sciences), Nup98 (rat MAb N1038; Sigma), Nup153 (rabbit Ab A301-789A; Bethyl Laboratories, Inc.), tubulin (murine MAb T9026; Sigma), and B23 (goat Ab SC-6013; Santa Cruz Biotech) were commercial, as were appropriate horseradish peroxidase-conjugated secondary antibodies (A0545, A2554, A5795, and A5420; Sigma). Proteins fractionated by SDS-PAGE were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore), which were blocked with Tris-buffered saline-Tween 20 (TBST; 20 mM Tris, pH 7.6, 140 mM NaCl, 0.5% Tween 20) containing 10% nonfat dry milk and then washed ($3 \times$) with TBST before incubation with appropriate primary antibodies (in TBST with 1% nonfat dry milk). The samples were washed again with TBST before incubation with secondary antibodies (in TBST with 1% nonfat dry milk). After final washes (3 times with TBST), the membranes were exposed to film in the presence of enhanced chemiluminescence substrate (GE Healthcare). Protein band intensities were quantitated by densitometry (Total Lab 100 software; Nonlinear Dynamics Ltd.).

RESULTS

2A^{pro} diversity. Completion of the reference strain sequences for all HRV-A and HRV-B and many HRV-C species has provided a fuller understanding of nonstructural gene conservation (28). Within and between species, particularly for HRV-A and HRV-C, the 2A^{pro} region occasionally undergoes intergenic recombination (18). Nonetheless, most of the reference strain 2A^{pro} proteases still parse into dominant clades and species, with general relationships similar to those of the genotype-defining capsid sequences (Fig. 1a). The average pairwise 2A^{pro} amino acid differences for HRV-A (12%), HRV-B (18%), and HRV-C (26%) are close to those of the respective P1 capsids (19%, 16%, and 25%) or full-length polyproteins (18%, 16%, and 26%). Within species, however, the values may vary considerably for any individual pair of genotypes. For example, 2A^{pro} proteases from A09 and A32 differ by only 1%. B70 and B91, or C03 and C06, differ by only 4%. More diverse pairs include A46 and A45 (39%), B69 and B84 (29%), and C02 and C03 (36%). Across species, the pairs can be closer than this (A25 and C10 differ by 32%) or much further apart (A101 and B99, at 69% difference). The diversity is distributed along virtually the whole protein. Only 28 of 142 amino acid positions have no substitutions in the known HRV database ($n = 144$), including the 7 residues required for enzyme catalysis and zinc coordination.

The biological consequences of the 2A^{pro} sequence breadth are unknown. To begin such comparisons, 6 enzymes were chosen as diverse representatives from available viruses (A89, B04, Cw12, and Cw24) or cDNAs (A16 and B14). Each corresponds to a separate dominant clade on the 2A^{pro} tree (Fig. 1A). The panel's amino acid differences range from 16% (A16 and A89) to 64% (A89 and B04 or Cw24 and B04) (Fig. 1C), with variability spanning most of the mapped structural elements of the enzyme (Fig. 1B). Despite these changes, all except B14 responded readily to bacterial expression and pu-

rification as soluble, stable proteins. The B14 enzyme needed a 2-step resuspension and refolding process before purification (41). Preparations across this panel produced active proteases, because purification required them to self-cleave from the VP1 fusion fragment when the construct was expressed. The purified enzymes gave unique bands on gels, with migrations consistent with individual molecular masses (range, Cw12, 15.6 kDa; B04, 16.3 kDa), charges (range, Cw12, 4.48 pI; B04 7.3 pI), and Pro/Gly contents (A89, 13%; B14, 21%) (Fig. 2). A polyclonal Ab raised to A16 2A^{pro} (1) cross-reacted with the A89 enzyme and also weakly with C15, an HRV-C species from a patient isolate with a 2A sequence that likely arose from recombination with an HRV-A species (5). A parallel serum raised to recombinant C15 did not react with 2A^{pro} outside the HRV-C species, reinforcing the idea that sequence differences across the 2A panel are so substantial that even polyclonal serum gives poor recognition beyond the species level (Fig. 2).

Cleavage of eIF4G. HeLa cell extracts, as the source of eIF4G, were reacted with enzyme equivalents (0.25 μ M) from the 2A^{pro} panel over a time course of 2 h. Cleavage was assayed by Western analysis using an Ab to the amino end of eIF4G and then quantitated by band densitometry. Each protease, except mutant A16_{C106A}, reacted with the extract substrates, giving the same product profiles (Fig. 3A), representing single cleavages within the common sequences but different-length amino regions of the 5 eIF4G isoforms (6). Within the panel, however, there were clear differences in the rates and final extents of cleavage, parameters which reproducibly segregated by species. Relative to tubulin loading controls, reduction in the initial eIF4G band followed the following pattern at all time points: HRV-A > HRV-C $\gg$ HRV-B (Fig. 3B). The B04 and B14 samples always lagged behind, with substrate turnovers less than half those of the others.

Cleavage of recombinant Nup62. During enterovirus (including HRV) infections, the cytoplasmic cleavage of eIF4G is followed by 2A^{pro}-dependent abrogation of nuclear cytoplasmic trafficking, brought about by proteolytic cleavage of one or more Nups within the central core of the NPC. Nup62, the smallest, most readily monitored (e.g., MAb 414) of known native NPC substrates, is cleaved by 2A^{pro} (30). Recombinant Nup62 linked to GST provided a soluble, readily purified substrate for direct comparative cleavage reactions. Examples of the beginning (15-min) and ending (8-h) cleavage profiles for the full enzyme panel illustrate the observed protease-specific banding patterns (Fig. 4A) characteristic of differential rates (Fig. 4B) and cleavage preferences. Clearly, 2A^{pro} was the effector here. Among the products were bands consistent with C- or N-terminal fragments from cleavage positions 103, 218, 247, and 298 within the Nup62 sequence, as described for A02 2A^{pro} (30), although until all bands from all enzymes are completely sequenced, these identifications should be considered preliminary. When measured by the decrease in the substrate band, Nup62 was turned over fastest by A16, A89, and Cw24. The HRV-A proteases gave identical processing profiles, generating the same size cleavage products with similar band intensities on stained gels. The HRV-C enzymes varied from this pattern, and from each other, in both the band appearance rate and final profiles. Some HRV-C fragments were of a size similar to those produced by HRV-A, but as a rule, such bands appeared at different times or in varying amounts. For exam-

FIG. 1. 2A^{pro} comparisons. (A) A relationship tree for 2A^{pro} from published HRV protein alignments (27) supplemented with recent HRV-C sequences (36) was calculated using neighbor-joining methods. The scoring matrix (*p* distance) considered amino acid similarities (e.g., F versus Y), as well as identities. Related clades (<10% *p* distance) were collapsed. The nodes show key bootstrap values (percentages of 2,000 replicates). (B) Ribbon diagram of A02 2A^{pro} shows the active-site residues relative to the di-tyrosine flap and opposing structural zinc ions. The backbone line width, a derivative of the PyMol *b* factor, superimposes the relative aligned sequence conservation of the 6 tested 2A^{pro} proteases along this structure. The thinner the line, the stronger the conservation in that region. (C) Alignment of A02 2A^{pro} sequence (GenBank caa26181.1) and structure elements (32) with cloned 2A^{pro}. The determined cDNA sequences for A16, A89, B04, B14, Cw12, and Cw24 match GenBank proteins aaa69862.1, ack37440.1, abf51184.1, aaa45758.1, abq51392.1, and abu62850.1, respectively, except for the strain-specific substitution D47G in A16 and 3 substitutions (R15A, T72A, and I113V) in Cw24, highlighted in boldface.

FIG. 2. Purified recombinant 2A^{pro} proteases (2 μg/lane) were fractionated by SDS-PAGE. (Top) Coomassie stain. (Middle and bottom) Western blots from similar gels probed with rabbit polyclonal serum raised to A16 or C15 recombinant 2A protein (1:5,000 Ab dilution).

ple, A16 and A89 produced an ~35-kDa fragment (103-N) rather early in the reactions. The HRV-C enzymes produced a similar-size fragment only at the end. Cw12, but not Cw24, showed an initial transient ~38-kDa product (218-C), but as with HRV-A, this product was gone after longer reactions. In the final samples (Fig. 4A, 8 h), neither HRV-C profile matched the other, and several fragments were clearly different from HRV-A. Multiple experiments confirmed these patterns as reproducible and characteristic of each enzyme. When the HRV-B 2A^{pro} proteases were tested, both proved inefficient. More than 50% of the full-length substrate remained uncleaved even after 24 h (data not shown). The few product fragments produced by B04 appeared to be subsets of the combined HRV-A and HRV-C profiles. These assays show that all 2A^{pro} proteases could act directly with Nup62, but at least 5 of the 6 tested enzymes had different cleavage prefer-

FIG. 3. Cleavage of eIF4G by 2A^{pro}. (A) HeLa cell extracts (40 μ l) were incubated with recombinant 2A^{pro} (16 pmol) at 35°C for up to 2 h. Loss of eIF4G signal over time was monitored by Western assays. Substrate band intensities (percent remaining [rem]) relative to buffer controls were normalized to tubulin. (B) The data in panel A, supplemented with a 5-min time point from the same experiment, were plotted.

ences along this recombinant substrate and produced unique processing patterns.

Cleavage within the NPC. Recombinant Nup proteins present different substrate challenges than native NPC. Isolated HeLa cell nuclei were reacted with the panel of recombinant

2A^{pro} proteases and then monitored for the loss of Nup signals using Western analyses with MAb 414, a reagent reactive to common FG regions within multiple Nups (Fig. 5). The control and A16_{C106A} lanes show the starting Nup profiles for undigested NPC. After 2 h with A16 and A89, Nup62 was gone from the nuclei, but for the HRV-B enzymes, Nup62 remained mostly intact, consistent with the poor reactivity shown by these 2A^{pro} proteases toward the recombinant version of the substrate. Nup153 was shifted lower on the gel in both HRV-B lanes. New reactive bands appeared between Nup153 and Nup62 in the HRV-A and HRV-B lanes that could be cleavage products from Nup153 or larger FG repeat nucleoporins (Nup214 or Nup358). Within the overall banding pattern detected by this MAb, the HRV-C lanes had been virtually cleared of most, if not all, Nup62 and Nup153. Only traces of the original proteins or their products remained.

Given the gross processing differences suggested by these reactions, it became important to document the rates at which individual enzymes selected their cohort of substrates. The experiment shown in Fig. 5 was repeated on a larger scale, collecting time points over 8 h and monitoring the cleavage of Nup62, Nup98, and Nup153 with antibodies developed to each protein. Similar to reactions shown in Fig. 4 and Fig. 5, Nup62 was never significantly diminished by either of the HRV-B enzymes (Fig. 6A and B). Even after 8 h of incubation, the levels of Nup62 that reacted with B04 and B14 2A^{pro} were little different than in control reactions. Therefore, Nup62 is not a preferred substrate for either of these enzymes. At the same time, the A16 and A89 enzymes readily cleaved Nup62, and in these assays, the rates were 2 to 3 times higher than those of HRV-C. A Nup62 antibody-reactive band with a molecular mass of ~54 kDa appeared on immunoblots during the course of reactions with HRV-A and HRV-C (Fig. 6A), suggesting all 4 enzymes may initially cleave this Nup at a common site, probably position 103. The cleavage fragment was then further processed by A16, A89, and Cw12, but not Cw24, because it disappeared during further incubation with these proteases. After 2 h of reaction, additional lower-molecular-mass products (~31 to 40 kDa [site 218]) were detected in reactions with HRV-A 2A^{pro} but not in reactions with HRV-C 2A^{pro} (Fig. 6A). The varied processing of Nup62 suggests the protease panel must target different sites in the Nup or perhaps targets the same sites but in different chronological order.

Nup98 and Nup153 were recognized and cleaved by all six recombinant proteases (Fig. 6A and B). For Nup98, 80 to 90% of the band signal was removed by incubation with HRV-A and HRV-C in less than 30 min, leaving an antibody-reactive product of ~60 to 65 kDa. HRV-B diminished Nup98 by only 50% within the same time, producing a slightly larger product (~65 to 70 kDa). None of these fragments were stable, and they gradually disappeared over time (Fig. 6A, 8 h), presumably because of additional 2A^{pro} cleavages. Nup153 was not cleaved as quickly as Nup98, but again, there was a distinct gradient in processivity rates: HRV-B $\gg$ HRV-C > HRV-A. For A16 and A89, a product band appeared at a molecular mass of ~120 kDa (Fig. 6A, 8 h) that was unique to these enzymes. A smaller product band of ~55 kDa was generated by HRV-C (not shown), but neither product was observed with HRV-B. In total, the nuclear-rate experiments demonstrated at the species level, and perhaps also at the genotype level, that the various

FIG. 4. Cleavage of Nup62 by 2A^{PRO}. (A) Recombinant GST-Nup62 (1 nmol) was incubated with the indicated recombinant 2A^{PRO} (0.2 nmol) as described in Materials and Methods. The samples were fractionated by electrophoresis and then visualized by Coomassie staining. Cleavage product identities, based on molecular weight (GST plus Nup62), are approximated according to sites mapped by Park et al. (30). "N" indicates that the cleavage product is the amino fragment from Nup62 cleavage at the designated site, and "C" indicates the carboxy fragment. (B) Gels similar to those in panel A recorded substrate loss over a time course of 8 h. The densitometry results for the substrate bands relative to control lanes were plotted.

2A^{PRO} proteases have dissimilar substrate priorities within the intact NPC. Differences in the early cleavage profiles (e.g., 30 min) were typically more profound than when the enzymes were allowed to react to completion (e.g., 8 h).

Cleavage during infection. Matched infections can be hard to achieve if viruses differ in receptor use or receptor affinity or have dissimilar specific infectivities (particle/PFU). The HRV-C species cannot (yet) infect current culture systems because their receptor use is certain to be different from the ICAM-1 or LDLR utilized by the other HRVs (5). The A89 and B04 viruses contributing to the 2A^{PRO} panel are from ref-

erence samples used infrequently in laboratories, and neither is well characterized for relative growth parameters. Therefore, comparative infection experiments in cells relied on A16 and B14, derivatives of cDNAs that use the same receptor, ICAM-1; are very well studied; and share similar plaque morphologies and specific infectivities (21, 22). HeLa cells infected with these viruses (MOI = 30) were probed for comparative rates of eIF4G and Nup turnover. For both viruses, the eIF4G bands diminished over time, giving rise to the canonical products characteristic of 2A^{PRO} cleavage (Fig. 7A). A16 infections turned over >90% of this protein in 8 h (Fig. 7B). B14 infections took almost 3 times as long to achieve the same effect, consistent with the much lower avidity toward the substrate by this 2A^{PRO} when tested *in vitro* (Fig. 3).

Likewise, the relative affinities for specific Nups mirrored those observed for these enzymes in cell-free assays. A16 infection reduced Nup98 and Nup62 to 45% and 65%, respectively, by 6 h postinfection (p.i.) while leaving ~85% of the Nup153 intact (Fig. 7A and B). The majority of this Nup was not turned over until 8 to 10 h p.i. For B14 infection, Nup153 was targeted more quickly (50% reduction by 4 h p.i.). The Nup98 and Nup62 bands diminished more slowly than after A16 infection or for Nup153 turnover, requiring 8 h and >12 h for similar reductions to ~50% levels. In this cell system, for both viruses, cytopathic effects (CPE) and cell rounding usually become evident at about 12 h p.i. Comparable protein reductions past this point may be due to cell death (loss from the plate) rather than proteolysis.

DISCUSSION

Viruses in the 3 species of HRV are linked to 50 to 85% of asthma exacerbation, wheezing illnesses in infants, high risk of childhood asthma, and many other types of morbidity associated with exacerbation of chronic lung diseases (13). To virol-

FIG. 5. Cleavage of NPC Nups by 2A^{PRO}. Samples containing isolated HeLa cell nuclei (6×10^6) and recombinant 2A^{PRO} (0.3 nmol) were incubated at 35°C for 2 h. Equivalent aliquots were fractionated by SDS-PAGE and then visualized after Western analyses (MAb 414).

FIG. 6. Differential cleavage of Nup153, Nup98, and Nup62 from isolated nuclei by recombinant HRV 2A proteases. (A) Nuclei were isolated from HeLa cells as described in Materials and Methods. Reaction mixtures containing nuclei (6×10^6) and recombinant 2A proteases (0.3 nmol) were incubated at 35°C for the indicated lengths of time, and the cleavage of Nup153, Nup98, and Nup62 was analyzed by Western blotting using Nup-specific antibodies. The nuclear protein B23 served as a loading control. Band intensities were quantitated by densitometry to determine the percentages of full-length protein remaining at the indicated times relative to buffer control. (B) The percentages of full-length Nup153, Nup98, and Nup62 remaining in reaction mixtures described in panel A after 0.5, 1, 2, 4, and 8 h were plotted to illustrate relative rates of Nup cleavage by different recombinant 2As.

ogists, it is not surprising that virus infections cause disease. The remarkable thing about rhinoviruses is the extraordinary range of etiologies triggered by these pathogens, which are habitually lumped into “HRV.” HRV-A and HRV-B alone comprise >100 serotypically distinct entities (28), with probably >50 more in the newly described HRV-C (36), but “serotype” only defines a host’s prior B-cell profile to a particular virus, not the accompanying phenotype of disease. It is what happens inside the cell that determines whether that cell will live or die, undergo necrosis or apoptosis, amplify the virus to high or low titer, activate any cell-to-cell signaling to modify the infection, and/or trigger cytokines and other immunological consequences.

It is the job of the first viral proteins produced in the first 2 to

3 h of a picornavirus infection, in the first infected cells, to shut off host defense response systems and to instigate others that may be advantageous for the virus. Viral proteases 2A^{pro} and 3C^{pro} carry out these functions for HRV. Enzyme 3C^{pro} is common to all members of the family *Picornaviridae*. As the workhorse of every polypeptide-processing scheme, it contributes significantly to viral replication mechanisms (31) and synergistically to the host shutoff activities instigated by 2A^{pro} or, e.g., the L proteins, which have similar antihost functions in other genera (11, 23). 3C^{pro} localizes to nuclei (2), cleaves crucial nuclear transcription factors (14), and participates (to a degree) in cellular cytokine regulation (12). These life cycle contributions are under investigation in a number of laboratories, and they are likely to show commonalities shared across many viruses.

FIG. 7. Cleavage of eIF4G and nucleoporins during HRV infection. HeLa cells infected with A16 or B14 (MOI = 30) were lysed in SDS gel loading buffer at the indicated times postinfection. Proteins were fractionated by electrophoresis and cleavage of eIF4G, and nucleoporins were analyzed by Western blotting. Tubulin served as a loading control. Band intensities were quantitated by densitometry to determine the percentages of full-length protein remaining at the indicated times postinfection relative to mock. (B) The percentages of full-length eIF4G, Nup153, Nup98, and Nup62 remaining in cells after the indicated times postinfection were plotted to illustrate the relative rates of eIF4G and Nup cleavage during A16 and B14 infections.

The 2A^{pro} proteases, however, are unique to the enteroviruses, including HRV. The full sequences for all known HRV-A and HRV-B species highlighted divergence within the protein in regions essential for substrate recognition (28, 38). If the 2A^{pro} divergence reflected altered substrate preferences *in vivo*, it could mean that the immunological alarms set off by any individual HRV, and the molecular consequences of those alarms, might have at their origin the efficacy with which 2A^{pro} (perhaps in combination with 3C^{pro}) did its jobs in the first round(s) of infection. If true, this hypothesis predicts measurable substrate preferences among divergent 2A^{pro} proteases. As a first test of this idea, we describe here the activities of 6 recombinant 2A^{pro} proteases representing all 3 HRV species. The HRV-C enzymes, and those from A89 and B04, are new to 2A^{pro} studies. Each was cloned and purified into a soluble, stable format. The various isoforms of eIF4G were cleaved by all enzymes in the panel (Fig. 3). The turnover rates, however, varied by more than 4-fold, with HRV-A > HRV-C >> HRV-B. These differences were reiterated during cell infection with A16 and B14 (Fig. 7), consistent with the idea that shutoff of host cap-dependent translation, and consequent control of the cell, is much slower for HRV-B.

The 2A^{pro} differences continued into studies with nucleoporins. Whether measured with recombinant Nup62 substrates or Nups provided by isolated nuclei (Nup62, Nup98, Nup153, and Nup214), the relative specificities and individual kinetics varied across the enzyme panel. The A16 and A89 proteases, those most similar in sequence (Fig. 1), proved similar in most assays. Their (putative) Nup62 sites were consistent with those described for A02 (30), including cleavage at positions 103,

218, 247, and 298. The Cw12 and Cw24 enzymes cleaved some of these sites (103 and 247[?]), but not others, and there were individual differences between these proteases in the rates and final profile patterns. Neither of the HRV-B enzymes was highly reactive with Nup62 in any substrate context. B04, but not B14, cleaved at most once (site 247), leaving nearly all the Nup62 intact (Fig. 4 to 6). The panel's overall proclivity for this Nup (HRV-A > HRV-C >> HRV-B) was consistent *in vitro* and *in vivo*, suggesting a targeted response programmed by these enzymes. Within the context of the NPC, Nup62 has a carboxyl-terminal NPC anchor. It traffics multiple karyopherins, including importin-β, transportin, Kapβ3, Crm1, and Ntf2, each of which is proposed to use different binding sites strung out along the length of the protein (35) to provide transport for cytokine-inducing transcription factors. Nup98 also has a carboxyl-terminal NPC anchor, and it is a required trafficking component for nuclear mRNA export via the Crm1 and TAP pathways (4, 10). This Nup was another preferred target for HRV-A and HRV-C (Fig. 6), but again, HRV-B, cleaved the protein about 4 times more slowly (HRV-A and -C > HRV-B) than the other 2A^{pro}. Rather, the best substrate for B04 and B14 was Nup153 (Fig. 6), which was reduced >80% in less than 1 h with these enzymes (Fig. 6). HRV-A and HRV-C also cleaved Nup153 (HRV-B >> HRV-C > HRV-A), but among these enzymes, there was a gradient of preference, depending on the virus strain (Cw12 > A16 and Cw24 > A89). Nup153 has an amino-terminal NPC anchor and is a required trafficking partner for various importin-αβ complexes, as well as Crm1 and Tpr (17, 35).

In total, the data for the 6 enzymes chosen as initial repre-

sentatives of their species showed variation in the site choices and cleavage rates for the tested cellular substrates. Composite amino- and carboxyl-terminal sequence maps for each observed cleavage fragment, including the (apparently) new Nup62 sites recognized by HRV-C, are under way. It is not yet clear whether the site preference data will correlate best with species or intraspecies clade-specific 2A^{pro} activity types. In the assays used here, enzymes from the same species tended to have similar, but not necessarily identical, activities. The interspecies differences were most striking when comparing HRV-A and -C with HRV-B. Except for Nup153, HRV-B was much less effective at targeting any of the tested substrates. Perhaps related to these observations, extensive patient data collected by the University of Wisconsin COAST (Childhood Origins of ASThma) study and associated analyses (3) indicate that naturally circulating HRV-B species are much less virulent than HRV-A and HRV-C in infant populations. Put simply, when wild-type viruses infect children, the HRV-B species are much less effective than their cousins and are less often associated with moderate to severe illness (3). In contrast, viruses in the HRV-C and the HRV-A groups are most frequently associated with severe respiratory disease (25, 33). The extensive panel of HRVs identified by the COAST study ($n = 367$) is currently being culled for particularly interesting isolates (HRV-A, -B, and -C), for both sequencing and additional comparative protease information (3).

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REFERENCES

- Amineva, S. P., A. G. Aminev, J. E. Gern, and A. C. Palmenberg. Comparison of rhinovirus infections in human primary epithelial cells and HeLa cells. *J. Gen. Virol.*, in press.
- Amineva, S. P., A. G. Aminev, A. C. Palmenberg, and J. E. Gern. 2004. Rhinovirus 3C protease precursors 3CD and 3CD' localize to the nuclei of infected cells. *J. Gen. Virol.* **85**:2969–2979.
- Bizzintino, J., et al. 2011. Association between human rhinovirus C and severity of acute asthma in children. *Eur. Respir. J.* **37**:1037–1042.
- Blevins, M. B., A. M. Smith, E. M. Phillips, and M. A. Powers. 2003. Complex formation among the RNA export proteins Nup98, Rae1/Gle2, and TAP. *J. Biol. Chem.* **278**:20979–20988.
- Bochkov, Y. A., et al. 2011. Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C. *Nat. Med.* **17**:627–632.
- Byrd, M. P., M. Zamora, and R. E. Lloyd. 2005. Translation of eukaryotic translation initiation factor 4GI (eIF4GI) proceeds from multiple mRNAs containing a novel cap-dependent internal ribosomal entry site (IRES) that is active during poliovirus infection. *J. Biol. Chem.* **280**:18610–18622.
- Castelló, A., J. M. Izquierdo, E. Welnowska, and L. Carrasco. 2009. RNA nuclear export is blocked by poliovirus 2A protease and is concomitant with nucleoporin cleavage. *J. Cell Sci.* **122**:3799–3809.
- Clark, M. E., T. Hammerle, E. Wimmer, and A. Dasgupta. 1991. Poliovirus protease 3C converts an active form of transcription factor III C to an inactive form: a mechanism for inhibition of host cell polymerase III transcription by poliovirus. *EMBO J.* **10**:2941–2948.
- Clark, M. E., P. M. Lieberman, A. J. Berk, and A. Dasgupta. 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C *in vivo* and *in vitro*. *Mol. Cell. Biol.* **13**:1232–1237.
- Cullen, B. R. 2003. Nuclear RNA export. *J. Cell Sci.* **116**:587–597.
- Devaney, M. A., V. N. Vakharia, R. E. Lloyd, E. Ehrenfeld, and M. J. Grubman. 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. *J. Virol.* **62**:4407–4409.
- Funkhouser, A. W., et al. 2004. Rhinovirus 16 3C protease induces interleukin-8 and granulocyte-macrophage colony-stimulating factor expression in human bronchial epithelial cells. *Pediatr. Res.* **55**:13–18.
- Gern, J. E. 2010. The ABCs of rhinoviruses, wheezing, and asthma. *J. Virol.* **84**:7418–7426.
- Ghildyal, R., et al. 2009. Rhinovirus 3C protease can localize in the nucleus and alter active and passive nucleocytoplasmic transport. *J. Virol.* **83**:7349–7352.
- Gradi, A., et al. 2003. Human rhinovirus 2A proteinase cleavage sites in eukaryotic initiation factors (eIF) 4GI and eIF4GII are different. *J. Virol.* **77**:5026–5029.
- Gustin, K. E., and P. Sarnow. 2002. Inhibition of nuclear import and alteration of nuclear pore complex composition by rhinovirus. *J. Virol.* **76**:8787–8796.
- Hase, M. E., and V. C. Cordes. 2003. Direct interaction with Nup153 mediates binding of Tpr to the periphery of the nuclear pore complex. *Mol. Biol. Cell* **14**:1923–1940.
- Huang, T., et al. 2009. Evidence of recombination and genetic diversity in human rhinoviruses in children with acute respiratory infection. *PLoS One* **4**:e6355.
- Lamphear, B. J., et al. 1993. Mapping the cleavage site in protein synthesis initiation factor eIF-4 gamma of the 2A proteases from human coxsackievirus and rhinovirus. *J. Biol. Chem.* **268**:19200–19203.
- Lee, W.-M., et al. 2007. A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. *PLoS One* **2**:e966.
- Lee, W. M., S. S. Monroe, and R. R. Rueckert. 1993. Role of maturation cleavage in infectivity of picornaviruses: activation of an infectious. *J. Virol.* **67**:2110–2122.
- Lee, W. M., W. Wang, and R. R. Rueckert. 1995. Complete sequence of the RNA genome of human rhinovirus 16, a clinically useful common cold virus belonging to the ICAM-1 receptor group. *Virus Genes* **9**:177–181.
- Lidsky, P. L., et al. 2006. Nucleo-cytoplasmic traffic disorder induced by cardioviruses. *J. Virol.* **80**:2705–2717.
- Liebig, H.-D., et al. 1993. Purification of two picornaviral 2A proteinases: interaction with eIF-4γ and influence on *in vitro* translation. *Biochemistry* **32**:7581–7588.
- Miller, E. K., et al. 2009. A novel group of rhinoviruses is associated with asthma hospitalizations. *J. Allergy Clin. Immunol.* **123**:98–104.
- Molla, A., A. V. Paul, and E. Wimmer. 1991. Cell-free, de novo synthesis of poliovirus. *Science* **254**:1647–1651.
- Palmenberg, A., et al. 2009. Sequencing and analysis of all known human rhinovirus genomes reveals structure and evolution. *Science* **324**:55–59.
- Palmenberg, A. C., J. Rathe, and S. Liggett. 2010. Analysis of the complete genome sequences of human rhinovirus. *J. Allergy Clin. Immunol.* **125**:1190–1199.
- Park, N., P. Katikaneni, T. Skern, and K. E. Gustin. 2008. Differential targeting of nuclear pore complex proteins in poliovirus-infected cells. *J. Virol.* **82**:1647–1655.
- Park, N., T. Skern, and K. E. Gustin. 2010. Specific cleavage of the nuclear pore complex protein Nup62 by a viral protease. *J. Biol. Chem.* **285**:28796–28805.
- Parsley, T. B., J. S. Towner, L. B. Blyn, E. Ehrenfeld, and B. L. Semler. 1997. Poly(rC) binding protein 2 forms a ternary complex with the 5'-terminal sequences of poliovirus RNA and the viral 3CD proteinase. *RNA* **3**:1124–1134.
- Petersen, J. F., et al. 1999. The structure of the 2A proteinase from a common cold virus: a proteinase responsible for the shut-off of host-cell protein synthesis. *EMBO J.* **18**:5463–5475.
- Renwick, N., et al. 2007. A recently identified rhinovirus genotype is associated with severe respiratory-tract infection in children in Germany. *J. Infect. Dis.* **196**:1754–1760.
- Rueckert, R. R., et al. 1979. Synthesis and processing of picornaviral polyprotein, p. 113–125. *In* R. Perez-Bercoff (ed.), *The molecular biology of picornaviruses*. Plenum Press, New York, NY.
- Ryan, K. J., and S. R. Wentz. 2000. The nuclear pore complex: a protein machine bridging the nucleus and cytoplasm. *Curr. Opin. Cell Biol.* **12**:361–371.
- Simmonds, P., et al. 2010. Proposals for the classification of human rhinovirus species C into genotypically assigned types. *J. Gen. Virol.* **91**:2409–2419.
- Sommergruber, W., et al. 1992. Cleavage specificity on synthetic peptide substrates of human rhinovirus 2 protease 2A. *J. Biol. Chem.* **267**:22639–22644.
- Sousa, C., E. M. Schmid, and T. Skern. 2006. Defining residues involved in human rhinovirus 2A proteinase substrate recognition. *FEBS Lett.* **580**:5713–5717.
- Strawn, L. A., T. Shen, N. Shulga, D. S. Goldfarb, and S. R. Wentz. 2004. Minimal nuclear pore complexes define FG repeat domains essential for transport. *Nat. Cell Biol.* **6**:197–206.
- Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**:1596–1599.

41. Wang, Q. M., et al. 1998. Enzymatic characterization of refolded human rhinovirus type 14 2A protease expressed in *Escherichia coli*. J. Virol. **72**: 1683–1687.
42. Wang, Q. M., R. B. Johnson, W. Sommergruber, and T. A. Shepherd. 1998. Development of *in vitro* peptide substrates for human rhinovirus-14 2A protease. Arch. Biochem. Biophys. **356**:12–18.
43. Yalamanchili, P., U. Datta, and A. Dasgupta. 1997. Inhibition of host cell transcription by poliovirus: cleavage of transcription factor CREB by poliovirus-encoded protease 3Cpro. J. Virol. **71**:1220–1226.
44. Yalamanchili, P., K. Weidman, and A. Dasgupta. 1997. Cleavage of transcriptional activator Oct-1 by poliovirus encoded protease 3C^{pro}. Virology **239**:176–185.