Encephalomyocarditis viral protein 2A localizes to nucleoli and inhibits cap-dependent mRNA translation

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Abstract

Panels of monoclonal antibodies were raised against viral non-structural proteins of encephalomyocarditis virus (EMCV) and used to probe infected cells in laser confocal microscopy experiments and Western analyses. Surprisingly, all Mengo virus and EMCV-infected cells showed strong targeting of protein 2A, 3B VPg, 3C pro, and 3D pol signals to cellular nuclei, in particular to nucleoli, from the earliest times of infection. Viral capsid proteins (1AB, 1C, and 1D) and other non-structural proteins (2B, 2C, and 3A) did not target nuclei and remained cytoplasmic throughout the infection. The cardio viral 2A protein (subject of this article) has an 143 amino acid sequence, terminating in a 19 amino acid COOH-terminal processing cassette (PCC) that participates in autocatalytic, co-translational primary cleavage of the viral polyprotein. The remainder of the 2A protein shares only limited similarity with other viral or cellular sequences, except for a short motif (KRPRPFRLP) near PCC resembling the nuclear localization signals (NLS) common to many yeast ribosomal proteins. Deletions within the EMCV 2A protein that impinge on this region have been reported to diminish the ability of virus to inhibit cap-dependent translation of cellular mRNAs [Svitkin et al., J. Virol. 72 (1998) 5811]. We have now observed that these same deletions prevented nuclear localization. Cellular expression of 2A protein from RNA transcripts or cDNAs confirmed that it does not require other viral proteins or activities for nuclear transport; even when expressed as a single protein, 2A protein effectively shuts off translation from capped reporter mRNAs. Within infected, transfected, or DNA vector-transformed cells, the 2A protein was always found in close association with the nucleolar ribosomal chaperone protein B23, which may help the traffic 2A into nucleoli like a surrogate ribosomal protein, by virtue of the putative nucleolar localization signal (NoLS). The data are consistent with a novel mechanism for virus-induced host protein shut off in cardioviruses, whereby 2A helps to upregulate the synthesis of new and modified ribosomes that have an inherent preference for internal ribosomal entry site (IRES)-dependent viral genome translation over cap-dependent host mRNA translation.

1. Introduction

RNA picornaviruses have evolved into remarkably robust and effective mechanisms to express their proteins at the expense of the host. Novel internal ribosomal entry sites (IRESs) allow these genomes to bypass normal translational requirements for 5' cap structures and efficiently lure the ribosomes into viral instead of cellular pathways. The captured ribosomes pass down a single, long ORF, creating polyproteins that are in reality, tandem linkages of all structural and enzymatic units necessary for rapid replication and virulent infection. The individual protein fragments are liberated co-translationally and post-translationally in an elaborate proteolytic cascade, that is, a defining feature of this family. No fewer than three virus-encoded catalytic entities are required for complete processing, none of which has an exact cellular analogue (reviewed in Palmenberg (1989) and Racaniello (2001)). The efficient processing scheme conserves essential space in a compact genome that apparently encodes only that cohort of protein, RNA, and regulatory elements, which directly contribute to a successful lifecycle.

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Encephalomyocarditis virus (EMCV) and Mengo-virus are prototypical members of the Cardiovirus genus of the Picornaviridae family. The special propensity of cardiovirus RNAs to facilitate efficient translation in cell-free extracts and the remarkable avidity of the processing cascade during these reactions, are hallmarks of cardiovirus genomes, and make these isolates exceptionally useful experimental subjects for molecular dissection of the picornavirus lifecycle and the processing cascade. With the exception of the co-translational primary cleavage mediated by protein 2A near the middle of the polyprotein, the remainder of the cardioviral cleavages do not have an obligate order (Parks and Palmenberg, 1987). Rather, the secondary processing, mediated by protease 3C\textsuperscript{pro}, is carried out by a series of mono- and bimolecular reactions with relative rates that seem to change over time (Palmenberg and Rueckert, 1982). The overall effect is a spectrum of mature proteins and partially cleaved precursors with compositions that shift progressively throughout infection. Some precursors, like L-P1-2A, P1, 1ABC, and 1AB are destined for final processing just before virion assembly. Others like 3AB, 3ABC, and 3CD play unspecified roles before and during viral RNA synthesis. Logically, the appearance of certain precursors may also be required to regulate the timing of critical viral events, such as the conversion of input genome RNAs from translation to replication functions, or as a trigger to initiate genome packaging. Over the years, as we tried to sort out these pathways, it became evident that new experimental tools were required to accurately follow the fate of precursors through the labyrinth of processing events, especially for proteins from the P2 and P3 regions, where the main replication functions are encoded.

We now report, the development of the first inclusive monoclonal antibody (mAbs) panel reactive against the full spectrum of cardiovirus proteins. Remarkably, the first use of these reagents highlighted several new protein pathways and pointed towards a completely unexpected and previously undescribed nuclear involvement by these proteins in the cardioviral lifecycle. Mature viral protein 2A and precursor 3BCD were found with unmistakable clarity and abundance in the nucleoli of EMCV-infected cells, even very early after the post-infection (PI) time. The experiments described below, and in the accompanying report (Aminev et al., this special issue, 2003), document these phenomena and their probable mechanistic consequences for the virus. The data support novel models that link viral-induced ribosome synthesis, IRES-dependent genome translation, the shut off of cellular cap-dependent translation, and cessation of pol-II-dependent mRNA transcription, during cardiovirus infection.

2. Methods

2.1. Viruses and cells

Recombinants vMwt (Mengo), vMC\textsubscript{0} (Mengo) and vEC\textsubscript{0} (encephalomyocarditis) have been described (Hahn and Palmenberg, 1995; Martin et al., 1996; species, encephalomyocarditis virus; genus, Cardiovirus; family, Picornaviridae). Mutant derivatives vE-2A\textsubscript{AS} and vE-2A\textsubscript{AI20} have deletions in the 2A coding-region relative to vEC\textsubscript{0} that remove 58 and 120 amino acids, respectively (Svitkin et al., 1998). The 19 amino acid COOH-terminal primary cleavage cassette (PCC) is intact and functional in both of these deleted viruses (Hahn and Palmenberg, 2001). Viruses were cultured in HeLa, L-929, or BHK-21 cells, at 37 °C under 5% CO\textsubscript{2}-air, using RPMI-1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum as described (Rueckert and Pallansch, 1981). Typically, subconfluent cell monolayers (5 × 10\textsuperscript{6} cells per 6 cm plate) were infected with virus at a multiplicity (m.o.i.) of 10 plaque-forming units (PFU) per cell.

2.2. Recombinant proteins

Isolation of recombinant Mengo 3C\textsuperscript{pro} and 3D\textsuperscript{pol} from Escherichia coli has been described (Hall and Palmenberg, 1996; Duque and Palmenberg, 1996). For convenience, the base numbering systems of vMwt (No. L22089) and EMCV-R (No. M81861) are used in all cloning descriptions. To isolate recombinant Mengo 2A protein, the gene (429 b) from pMC\textsubscript{0} (b 3462-3890) was amplified by PCR. One primer contained an engineered Nde I site, an ATG codon, and 19 nucleotides derived from the 5’ end of the 2A-coding region. The second primer contained nucleotides complementary to the 3’ end of 2A region, the complement of a TAG codon, and a Bam HI site. The ampiclon was subcloned into pET-41b (Novagen), transformed into E. coli (strain BL21-DE3-pLysS, Novagen), and the bacteria were amplified in 2 × YT broth (37 °C), supplemented with kanamycin (15 mg/ml) and chloramphenicol (30 mg/ml). After induction (5 h, with 1 mM isopropylthio-β-d-galactoside), the cells were pelleted and then resuspended in buffer A (1/10 volume, 50 mM Tris–HCl, pH 8.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% deoxycholate), lysed by freeze-thaw and subject to centrifugation (27,000 × g, 15 min). The pellet was washed twice (buffer A). The inclusion bodies were collected by centrifugation (12,000 × g, 5 min), then resuspended in buffer B (0.1% Triton X100, 100 mM NaCl, 6 M urea, 50 mM Tris–HCl, pH 8.0, 1 mM PMSF). Insoluble material was removed (12,000 × g, 5 min), and the supernatant was dialyzed against TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.1% Triton X100), then concentrated (Centricon-10, Amicon).
Recombinant Mengo 2B protein (b 3891–4343) was produced in E. coli from a pET-41b plasmid (Novagen) that had been engineered to contain the pMC0 2B gene (450 b), in a manner similar to that described above. Recombinant Mengo 2C protein (b 4344–5318) was also produced from pET-41b, but as a fusion protein that linked a GST–His–Stag segment to the N-terminus of the viral sequence (2C-specific primers had added Nco I and Bam HI sites). The 2C-containing protein was isolated from disrupted cells and purified by fractionation on a His-column (Novagen). EMCV-R proteins 3A and 3AB (b 5391–5654 and b 5391–5714, respectively) were expressed using procedures similar to those described for 2A and 2B, except the vector, pET-11a (Novagen), was used. Bacteria, harboring these plasmids, were grown in M9 media supplemented with glucose (0.2%) and rifampicin (1 mM, Sigma). The 3A- and 3AB-containing inclusion bodies were resuspended in buffer B1 (0.5% Triton X100, 500 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1 mM PMSF) before clarification and dialysis.

2.3. EMCV replicons

Mengo replicons which express active firefly luciferase after RNA transcript transfection of HeLa cells have been described (Duque and Palmenberg, 2001). A similar EMCV-based replicon (pE-luc) was created by replacing an Sph I to Ava I fragment of pEC9 (b 2426–3509 in 1C–1D coding region; Hahn and Palmenberg, 1995) with an in-frame firefly luciferase gene (from plasmid pGL2, Novagen), proceeded by an autocatalytic PCC derived from the pMC0 2A–2B junction (84 b of 2A, 30 b of 2B). Translation of T7 RNA transcripts from this replicon produced a full-length, active luciferase enzyme with 10 additional viral amino acids at the N-terminus (from the Mengo PCC), and 29 additional viral amino acids at the C-terminus (from EMCV 1D). A derivative, pE-luc-2AASS, deleted 174 bases (b 3777–3951) encoding 58 amino acids (Beretta et al., 1996) from the middle of the EMCV 2A gene. A second derivative, pE-luc-Δ3Dpol, had a 555 base deletion (b 7177–7732) that effectively removed the active site of viral 3Dpol (Eco47 III–Mlu I fragment). Proteins directed by these transcripts were inactive for viral RNA synthesis. Luciferase assays in extracts from transfected HeLa cells were as described (Duque and Palmenberg, 2001).

2.4. Eukaryotic 2A expression vectors

The Mengo 2A and 2AB genes were engineered into vectors designed to evaluate protein expression in HeLa cells after RNA transcript transfection. The vectors were based on plasmid pCITE-4b (Novagen), which contains an EMCV IRES under the control of a T7 polymerase promoter. Appropriate viral amplicons encoding 2A or 2AB were derived from pMC0 using the Nde I- and Bam HI-containing primers described above. Gel-purified fragments were ligated into pCITE DNA that had been digested with these enzymes. When translated in reticulocyte extracts (Shih et al., 1979), T7 transcripts from pCITE-2A and pCITE-2AB produced viral proteins of the expected size and immunogenicity, and PCC within 2AB cleaved to near completion (results not shown).

The Mengo 2A gene was also engineered into a DNA transformation vector (Pmin CMV), where protein expression was under the control of an inducible, minimal cytomegalovirus promoter (pTRE2hyg, Clontech). PCR reactions included one primer with a Bam HI segment linked to a 5′ fragment of EMCV IRES, and another primer complementary to the 3′ end of the 2A gene, linked to an Mlu I segment to amplify IRES-2A sequence from within pCITE-2A. The amplicon was used to replace the Bam HI–Mlu I fragment of pTRE2hyg. When transformed into HeLa cells and induced by tetracycline (2 μg/ml, according to manufacturer’s instructions), pIRES-2A produced (capped) transcripts that directed 2A translation under the control of EMCV IRES.

The EMCV 2A gene and derivative 2AASS were engineered into bicistronic, luciferase-containing plasmids under the control of the immediate early promoter sequence of cytomegalovirus (P CMV IE ). Commercial plasmid pIRES (Clontech) was used to link this constitutive CMV promoter to a firefly luciferase gene, followed by an intact EMCV IRES and a multiple cloning site (MCS). The 2A and 2AASS segments from pEC9 and pECO-2AASS were amplified using flanking primers containing Xba I or Not I sequences, then ligated into the pIRES MSC, after digestion with appropriate enzymes. The pIRES-luc (control), pIRES-luc-2A and pIRES-luc-2AASS plasmids (5 μg) were transformed into HeLa cells (5 × 10⁶ per plate) with transfectin (Qiagen), then incubated (37 °C, 5% CO₂-air) in the presence of neomycin, following manufacturer’s instructions. Luciferase assays in extracts from transformed cells were as described (Duque and Palmenberg, 2001).

2.5. Antibodies

Murine monoclonal antibodies (mAbs) raised against recombinant Mengo 3Dpol and capsid protein 1CD were generously supplied by Dr. H. Duque, Dr. V. Frolov, and Dr. O. Frolova (Duque and Palmenberg, 1996). Murine monoclonal and polyclonal antibodies to Mengo 2A, 2B, 2C (GST–His–Stag fusion), 3A, 3AB, and 3Cpro were developed from recombinant proteins (above) according to the described methods (Duque and Palmenberg, 1996; Hall and Palmenberg, 1996). To raise
anti-3B antibodies, a water-solubilized synthetic peptide (GPYNETARVPKGTLQDLIQ) corresponding to the complete EMCV 3BVPg protein (b 5655–5714) was conjugated with KLH (keyhole limpet hemocyanin) and used to immunize mice (40 µg, over 6 doses, during 18 weeks). Antibody reagents raised against each viral protein were tested for high-titer ELISA reactivity with the corresponding recombinant protein. The antibody subtypes (Ig) produced by each line (hybridoma or ascites) were identified with commercial test kits (Sigma). Goat polyclonal antibodies to nucleolar-specific proteins, B23 and C23, were purchased (Santa-Cruz Biotechnology, Inc.).

2.6. Immunodetection of proteins

Western assays used cells harvested at appropriate times of PI. The cells were washed with phosphate-buffered saline, lysed by freeze-thaw (3 ×), and the clarified supernatants were fractionated by SDS-PAGE, then blotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore) as described (Duque and Palmenberg, 2001). Bands with positive reactions against the appropriate antibodies (typically, 1:2000 dilution of ascites), were visualized by chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Inc.), after secondary reactions with appropriate anti-mouse antibodies, conjugated with horseradish peroxidase (1:2000 dilution, Sigma). Mobility shift assays with viral proteins by non-denaturing 4% PAGE were similar to those described (Kiessig et al., 2001; Stern and Frieden, 1993). Briefly, recombinant 2A protein (30 ng) and nuclear extracts or whole cell lysates from HeLa cells (20 µl, Promega) were co-incubated in buffer (20 mM HEPES, pH 8.0, 100 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 0.1% Triton X100, and 1 mM DTT) for 1 h at 30 °C. The samples were fractionated by non-denaturing PAGE (20 °C, TBE buffer, 200 V), transferred to membranes, and the bands were visualized by Western assay (Ab-B23 or mAb-2A), using ECL (anti-goat or anti-mouse secondary antibodies, respectively).

2.7. Immunofluorescence microscopy

HeLa or L-929 cell monolayers were grown on coverslips to 50–70% confluence then infected (10 PFU/cell), transfected (1.5 µg RNA per 5 × 10⁶ cells), or transformed (5 µg DNA per 5 × 10⁶ cells) as described above. At appropriate intervals (37 °C under 5% CO₂-air), the cells were rinsed with phosphate-buffered saline, fixed with paraformaldehyde, permeabilized (0.3% Triton X100), then incubated (1 h, 20 °C) with the desired primary antibody (1:2000 dilution in blocking solution), as described (Amineva et al., submitted for publication). After reaction with a corresponding secondary antibody, the slips were mounted (Vectashield mounting medium, Vectorlab) and label location was visualized by laser-scanning confocal microscopy. Image capture used MCR 1024 LaserSharp software (Bio-Rad, Inc.). For double-label experiments, primary antibodies raised in different species (e.g. mouse and rabbit) were selected, and the samples were developed with appropriate and corresponding secondary antibodies (1:100 dilution) conjugated with fluorescein isothiocyanate (FITC) or Texas Red (Santa-Cruz Biotechnology, Inc.). RNA labels incorporated fluorescein-12-UTP (Sigma) into permeabilized cells before the paraformaldehyde fixation step, as described previously (Aminev et al., this special issue, 2003; Amineva et al., submitted for publication). A fluorescent-tagged wheat germ agglutinin (WGA, Molecular Probes, Inc.) was used to highlight and identify Golgi and nuclear membrane locations. SYTOX stain (Molecular Probe, Inc.) was used to localize dsDNA within cells. Rabbit polyclonal serum against ribophorin II was generously provided by Dr. Anant Menon.

3. Results and discussion

3.1. Recombinant viral proteins

We have described the cloning and isolation of recombinant Mengo 3C/pre and 3D/pool, and the characterization of murine monoclonal antibodies raised against these proteins (Duque and Palmenberg, 1996; Hall and Palmenberg, 1996). Using similar procedures, the genes for 2A, 2B, 2C, 3A, and 3AB were cloned into bacterial expression vectors. The 2C gene was linked to a GST–His–Stag fragment in a construction, which facilitated subsequent protein solubility and isolation, but the other proteins were synthesized as unmodified, non-fusion sequences. Most of the expressed material was produced in inclusion bodies, which were readily solubilized by detergent and dialysis. A typical yield was about 100 mg of protein per liter of culture. After verification of protein size and purity by SDS-PAGE, the preparations were inoculated into mice. To complete the panel, a synthetic peptide with the Mengo 3BVPg sequence was inoculated in parallel. Animals which seroconverted to their respective proteins (ELISA) were exsanguinated (polyclonal sera) and their spleens were used to develop hybridomas producing monoclonal antibodies and subsequent high-titer ascites (Duque and Palmenberg, 1996). Each reagent was tested for ELISA reactivity with its recombinant protein and also in Western assays with lysates from Mengo-infected HeLa cells, so the precursor recognition capacity could be characterized. As an example, five IgG-producing hybridoma lines were generated to 2A (5A12, 5F5, 4D10, 3A6, and 1C7), each of which reacted strongly with recombinant protein in ELISA, immunoprecipi-
tated its targets from infected cell lysates, and stained infected cells by confocal microscopy. But only two of these 2A mAbs (5A12 and 5F5) gave positive signals in Western assays with infected lysates. When used at appropriate concentrations (typically, 1:2000 dilution of ascites), only a few mAbs within the entire panel of new reagents showed any reactivity with cellular proteins. Nevertheless, care was taken in every experiment to include uninfected cell samples for the identification of spurious signals.

The Western profiles of selected mAbs are shown in Fig. 1A. Although cardiovirus precursor identification is well characterized for this gel system (Palmenberg and Rueckert, 1982; Parks et al., 1986), in all cases, the band assignments were confirmed by stripping and reprobing the blots with various combinations of mAbs (results not shown). Kinetic analyses of viral proteins synthesized in EMCV-infected cells will be published in detail elsewhere (Aminev and Palmenberg, in preparation), but to summarize, the mAbs proved quite adept at detecting “missing-link” precursors, like 2C3AB, 2C3A, 3BCD, 3BC, and 3ABC. Moreover, some mAbs also detected the surprising presence of stable populations of dimers or other multimers for 2A (16.7 kD, PI of 9.2) and 2B (16.5 kD, PI of 7.9). An infection time course (Fig. 1C) indicated that by 3 h PI, nearly 10% of the 2A signal migrated as a P1-2A capsid precursor and another 10% migrated in the position of a 34 kD complex on SDS-PAGE. This complex was not reactive to mAbs against the flanking proteins 1D or 2B (results not shown), and was, therefore, not derived from a previously undescribed precursor. A gel fractionating the recombinant 2A protein (Fig. 1C, r2A lane) also had the 34 kD band, consistent with its identification as an SDS-

![Fig. 1. Panel A: Antibody activity. A map of EMCV genome shows the protein order within polyprotein reading frame. HeLa cells infected with vEC9 (m.o.i. of 10) were harvested (4 h PI), and analyzed for viral protein content in Western assays with mAbs to 2A (5A12), 2B (3G3), 2C (9H11), 3A (3D2), 3BVPg (2E4), 3Cpro (1D12), and 3Dpol (H. Duque). Panel B: Cell staining. The same mAb panel (and mAb-1CD from V. Frolo) was used to stain infected cells for visualization by confocal laser microscopy using an anti-mouse, FITC-conjugated secondary antibody. Panel C: Protein 2A accumulation. Infected HeLa cells were harvested at the indicated times, fractionated by SDS-PAGE, then probed by Western analyses using mAb-2A (5A12) or Ab-2A (murine polyclonal serum). Recombinant 2A (r2A) is included as a marker. Panel D: L-292 cells were infected with vMwt (m.o.i. of 10), and harvested at the indicated times for confocal microscope visualization, using the above mAb-2A.](image-url)
resistant dimer form. Notably, the Mengo 2A sequence does not encode cysteine, but it does predict a helix–loop–helix domain, similar to a type that commonly mediates dimerization among certain transcription factors (Murre et al., 1989), or dimerization of the herpesvirus MEQ protein (Liu et al., 1997). Recombinant 2B protein (results not shown), or 2B from infected cells, likewise had a 36 kD form that probably also represented dimers.

3.2. Protein 2A localizes to nucleoli

Having identified the cohort of viral precursors (or multimers) that gave rise to immunogenic signals, the next step was to identify within cells, their preferred location. The replication of infectious picornaviruses is a cytoplasmic process. Indeed, infectious poliovirus can be completely synthesized in cell-free extracts (Molla et al., 1991), and this virus will replicate in enucleated cells (Detjen et al., 1978). Electron microscopy studies with poliovirus (using mAbs to 2C and 3Dpol) have confirmed repeatedly that genome translation and replication take place in cytoplasmic foci where the smooth and rough endoplasmic reticulum (ER) are in near conjunction (Bienz and Egger, 1995; Bienz et al., 1983, 1992; Egger et al., 2000). Our first microscopy experiments with cardiovirus mAbs were in agreement with these findings, in that signals from capsid proteins (1CD) and non-structural proteins 2B, 2C and 3A (Fig. 1B) colocalized with ribophorin II and WGA markers for ER and Golgi (e.g. Fig. 2D). Signals from 2A, 3BVPg, 3Cpro, and 3Dpol also colocalized with ER. However, these proteins showed additional, unmistakable targeting to nucleoli as well (Fig. 1B). Even at the earliest times of EMCV infection (vEC9), the nucleoli glowed brightly from reactions with mAbs to 2A, 3Cpro, 3Dpro, or 3BVPg. The same results were evident with vMwt virus in L-929 cells (Fig. 1D) or BHK-21 cells (Fig. 2B). A simple time course of 2A localization during vMwt-infection of L-929 cells (Fig. 1D) indicated that the earliest punctate nuclear foci (2.5 h) were progressively replaced with dense nucleolar signals (4–6 h), and finally by diffuse cytoplasmic labeling in addition to the nucleolar stains.

Fig. 2A (top 4 panels) shows a HeLa cell infection by vEC9 at 2, 4, and 6 h PI. This 2A antibody was not reactive to mock-infected cells (top panel), but in infected cells, the signal colocalized with a pulse-labeled FITC-UTP stain that was added to mark the sites of ongoing RNA synthesis. The densest nuclear RNA stains (green) identify transcriptionally active nucleoli and are also the sites where ribosomal chaperone protein B23 (nucleophosmin) was significantly concentrated (Fig. 2B). The panels in Fig. 2B are from vMwt-infected BHK-21 cells taken at 3, 4.5, and 6 h PI. EMCV productively infects only about 10% of these cells, compared to nearly 100% of HeLa cells (Martin et al., 2000), so the antibody staining also highlights the obvious contrasts between infected and uninfected cells within the same field. In this case, the B23 nucleolar staining (red) is evident in all cells, making the cytoplasmic (green) and nucleolar (yellow) distribution of 2A in the infected cells, all the more apparent. Similar experiments with antibodies against nucleolin C23 protein (Li et al., 1996) had nearly identical patterns (results not shown).

As described above and shown in Fig. 1B, the localization of 2A to nucleoli of infected cells was not unique to this protein. Protease (3Cpro), polymerase (3Dpol), and VPg (3BVPg) antibodies gave similar signals in all infected cells. The P3-region protein reactions are described more fully in the accompanying paper (Aminev et al., this special issue, 2003), but in order to learn whether these signals were co-dependent, it became necessary to express 2A from various genome contexts within cells. Recombinant EMCV with 2A deletions are known to be infectious as long as the COOH-terminal 19 amino acids of 2A (PCC) are left intact to catalyze the primary cleavage of the polyprotein (Hahn and Palmenberg, 2001). Viruses vE-2AAsS and vE-2AAsD120, for example, grow well in cells, albeit with small plaque phenotypes because of their inability to shut off host protein synthesis or host mRNA transcription (Svitkin et al., 1998). After infection with these mutants, mAb-2A (5A12) which recognizes a PCC epitope, localized the remaining 2A fragment(s) to ER, but not the nucleolus, confirming that nuclear targeting required an intact 2A (Fig. 2A, bottom panel). Other viral proteins were not essential to this process because transfection with RNA transcripts linking the 2A gene to a viral IRES (pCITE-2A), also resulted in strong nucleolar signals (Fig. 2C). Similar transcripts expressing 2AB (pCITE-2AB), showed that only the 2A protein became nuclear, while 2B moved to the Golgi.
Fig. 2.

A. vEC$_9$ in HeLa Cells

- FITC-UTP
- mAb-2A
- Merged

Mock

3 h

4 h

6 h

vEC$_9$-2A$_{58}$ in HeLa Cells

4 h

B. vMwt in BHK-21 Cells

- Ab-B23
- mAb-2A
- Merged

3 h

4.5 h

6 h

C. pCITE-2A Transfection of HeLa Cells

- Ab-B23
- mAb-2A
- Merged

6 h

24 h

D. pCITE-2AB Transfection of HeLa Cells

- Ab-B23
- mAb-2A
- Merged

6 h

24 h

24 h

WGA

24 h

Ab-Ribophorin II

E. pIRES-2A Transformation of HeLa Cells

- CMV
- mAb-2A
- Merged

Mock

SYTOX

24 h

48 h
(WGA stain) or ER (Ab-ribophorin II) as soon as the fragments were separated by the co-translational activity of the encoded PCC (Fig. 2D). In yet another construction, 2A expressed after tetracycline induction of a cDNA containing a CMV promoter (pIRES-2A) also localized to nucleoli almost immediately after synthesis (Fig. 2E). Interestingly, under these conditions, the cells rounded up and died within 24–48 h of induction, presumably from the toxic effects of 2A overexpression (Fig. 2E, bottom panel).

3.3. Protein 2A and B23

The sequence and structural elements that allow mammalian proteins to shuttle into nuclei and nucleoli are not very well understood. No cardiac proteins, including 2A, have canonical nuclear localization signals (NLS), that exactly match the three classical types described for eukaryotic proteins using the importin α or importin β pathways (Michael, 2000; Hicks and Raikhel, 1995), nor do the viral sequences suggest an obvious small nuclear RNA (snRNA) binding motif that might easily explain their targeting. On the other hand, 2A does have a rather basic amino acid profile (18% H+K+R, pI of 9.23), and contains at least one segment resembling the [KR][KR][X]10[3 of 5: KRHW] sequence that defines about 50% of known nuclear proteins in eukaryotic cells (Michael, 2000). Moreover, a subset of this segment (Fig. 3) is a reasonable match with the nuclear targeting pattern, common to many yeast ribosomal proteins (YRP, reviewed in Stuger et al. (2000)). Mammalian r-proteins use a specialized nuclear import pathway that does not involve importin α. YRP-NLS, which may or may not have a functional analog in mammals, follows the general pattern of (K/R)3X14, preceded or followed by Gly or Pro. All known cardiac 2A proteins have this pattern just upstream of PCC, although it is deleted in the v-E2A<sub>AS</sub> virus. Site-directed mutagenesis studies are currently underway to map the function of this region in 2A, and determine whether this small motif is uniquely responsible for nuclear targeting.

The nucleolar signals within 2A are yet another unknown. Nucleoli, of course, are the genome sites where pol-l transcribes dense clusters of rRNA genes into a 45S pre-rRNA. These ribosome factories splice pre-rRNA into 18S, 28S, and 5.8S fragments as the 40S and 60S ribosomal subunits are assembled on site. Ribosomal 5S rRNA, synthesized by pol-III, and ribosomal proteins translated in the cytoplasm, are shuttled into complex nucleolar scaffolds as the ribosomes are built (Melese and Xue, 1995). The most curious observation about nucleoli in Mengo-infected cells was that they never shut off their synthesis. Nucleolar antibodies (B23 and C23), viral mAbs (2A), and fluorescein-UTP labels showed progressively brighter foci throughout the infection and continuous rRNA synthesis (Fig. 2A). In contrast, during polio or rhinovirus infection, nucleoli become diffuse and disassembled (Amineva et al., submitted for publication), and in fact, the whole nuclei eventually become physically indistinct, presumably as the virus perverts essential pathways for transport and repair (Waggoner and Sarnow, 1998).

The requirements for protein accumulation in nucleoli are still unclear, although binding to nucleolar proteins or nucleolar RNAs (rRNA) is probably a common theme (Valdez et al., 1994; Li et al., 1996). In this regard, the close association between B23 and cardioivirus 2A (or the 2A dimer?) by microscopic localization, might

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**Fig. 3.** Cardioivirus NLS. A representative alignment (Palmenberg and Sgro, 2001) of cardioivirus sequences near the COOH-termini of the viral 2A proteins is shown. GenBank accession numbers for representative strains of the Theloviruses (tme) and Encephalomyocarditis viruses (emc and mengo) are given. Co-translational primary cleavage of each viral polyprotein occurs between the Gly-Pro (G/P) of the PCC (EMCV-R: YAGYFADLLIHDITNPFP). The consensus nuclear localization sequence found in several yeast ribosomal proteins (Stuger et al., 2000) is indicated, and the potential viruses are highlighted.
not be coincidental (Fig. 2B–D). During HIV infections, B23 interacts with Rev, Rex, and Tat viral proteins and helps to direct them into nucleoli (Szebeni et al., 1997; Stauber and Pavlakis, 1998; Hiscox, 2002; Adachi et al., 1993). As a preliminary test for a similar association, we added recombinant 2A to nuclear extracts from uninfected HeLa cells and observed a shift in the B23 immunogenic signal towards the top of the gel when the samples were fractionated under non-denaturing conditions (Fig. 4A). The gel shift required the presence of r2A (lane 3 vs lane 4, or lane 5 vs lane 6), but was not sensitive to treatment with RNaseA (lane 3 vs lane 5). The slower migrating complexes, whether formed by mixing uninfected extracts with r2A (Fig. 4A), or fractionated from infected cells (Fig. 4B), were co-reactive with antibodies against 2A as well as B23, suggesting a direct interaction, or reaction within a common heterologous complex.

In parallel experiments, two different mAb-2A samples (5A12, 5F5) were tested for their ability to immunoprecipitate B23 from infected cell extracts (Fig. 4C). Both extracted an Ab-B23 reactive band of the same size as authentic B23 (lane 1, marker) from infected cells (lanes 4 and 5), but not from uninfected cells (lanes 2 and 3). The reciprocal experiment showed that Ab-B23 was able to immunoprecipitate 2A dimers from cell extracts after 4 h (lane 6) of infection. Although there is strong evidence for a direct interaction between B23 and 2A (or the 2A dimer?), these collective results could also indicate a mutual participation in one or more complexes of cellular proteins (e.g. ribosomes?). Nevertheless, our preferred working hypothesis is that B23 may be required to chaperone 2A into nucleoli during infection in the same manner it chaperones ribosomal proteins or HIV proteins. If true, 2A probably encodes YRP-type NLS to allow de facto behaviour as a ribosomal protein mimic, thereby facilitating quick and efficient nucleolar transport. Ideas like this, however, will clearly require many more experiments before we can prove these points, or unravel the relevant mechanistic pathways.

### 3.4. Protein 2A shuts off cap-dependent translation

Why should a virus need such an unusual nuclear function, or nucleolar-targeting protein in the first place? RNA viruses live short, intense predominantly cytoplasmic lifecycles. Within 6–8 h, they infect, translate, synthesize RNA, package their progeny, and exit the cell, all while in direct competition with host for metabolic resources. Preferably, they can simultaneously avoid activating the plethora of host-encoded antiviral defense systems that could trigger an abortive infection. A virus can gain an edge in this competition if it inhibits detrimental cellular responses or effectively sequesters metabolic resources. Picornaviruses do both. Observations dating back at least 40 years report a marked shut off of host mRNA synthesis following Mengo infection of L-929 cells (Baltimore and Franklin, 1962). Careful measurement of the DNA-dependent RNA polymerase levels (pol-II) in infected cells showed that these enzymes were not damaged or denatured, but nonetheless were unable to initiate new mRNA synthesis in the manner of mock-infected cells (Apriletti and Penhoet, 1978; Baltimore and Franklin, 1962). At the same time, there was a
strong, parallel shift from cap-dependent (host) to IRES-dependent (viral) translation, during the EMCV infectious cycle (Svitkin et al., 1978). More recently, Nahum Sonenberg’s group conducted an elegant series of experiments clearly connecting EMCV 2A to the shut off of host protein synthesis during viral infection (Svitkin et al., 1998). Those experiments implicated the PI3 kinase-FRAP signaling pathway in this process, but did not determine the precise mechanism by which cap-dependent inhibition was achieved. The 2A proteins of polio- and rhinoviruses are proteases, which, among other duties, cleave translational factor eIF-4G to inactivate the host’s cap-binding complex (Krausslich et al., 1987; Haghigat et al., 1996; Liebig et al., 1993). In aphthoviruses, the leader protein carries out similar cleavages (Guarner et al., 1998). By inactivating eIF-4G, these viruses effectively prevent host mRNA translation while they themselves continue to translate by virtue of their IRESs. But in EMCV-infected cells, eIF-4G is not cleaved, 2A is not a protease (nor is the leader), and the shut off of host protein synthesis, while clearly evident, is neither as rapid nor as extensive as that caused by polio (Jen et al., 1980).

To resolve these questions, we are only now in the process of probing cells for specific phenotypes associated with the cardiovirus 2A protein, in the absence of infection. The pIRES-2A cDNA described above is certainly a useful tool, because of the rapidity and totality with which the induced 2A kills cells. On the other hand, rapid cell death can sometimes make it even harder to tease apart the lethal pathways if there is an inadvertent triggering of unwanted apoptotic responses. Among our more subtle approaches are new EMCV cDNAs encoding RNA replicons or bicistronic reporter mRNAs. A replicon is a non-infectious viral RNA transcript, where a portion of the capsid-coding region has been replaced by a reporter gene, typically luciferase (Fig. 5A). Reporter activity after transfection is usually sensitive enough to discriminate between input transcript RNA translation (1–4 h post-transfection) and replicated progeny RNA translation (4–8 h post-transfection). In the case of EMCV replicons harboring the 2AASS deletion, we found both translational phases that were somewhat diminished relative to wild-type (Fig. 5B), indicating that the full-length form of the protein was providing a metabolic boost to the entire replication cycle, even from the earliest times of expression. A wild-type 2A then, capable of nucleolar translocation and host translational shut off, confers a competitive advantage to its virus or replicon. To determine whether the 2A protein acted alone in this process, special bicistronic cDNAs were engineered, placing a luciferase reporter gene under the translational control of a 5’ cap, and linked to a downstream 2A gene under the translational control of an EMCV IRES (Fig. 5A). Consequent transformation of cells resulted in ample expression of luciferase from control cDNAs (pIRES-luc), but co-expression of 2A from the downstream cistron of the same RNA decreased the cap-dependent luciferase activity by nearly 50-fold over the course of 48 h (Fig. 5C). Of particular interest was the additional observation that co-expression of 2AASS in place of the wild-type 2A, was also partially inhibitory to cap-dependent translation (about threefold reduction in luc activity), even though this protein was excluded from nucleoli, and must necessarily have exerted its residual function(s) only from the cytoplasm.

4. Conclusions

The discovery of a significant nuclear lifecycle for cytoplasmic RNA picornaviruses took us by surprise. Previous electron microscope studies with poliovirus (using mAbs to 2C and 3Dpol), carried out over the past 20 years (Bienz and Egger, 1995; Bienz et al., 1983; Egger et al., 2000) never hinted at a significant nuclear localization during picornaviral replication. Yet even at the earliest times of Mengovirus or EMCV infection, the nucleoli of infected cells glowed like little stop-lights when reacted with mAbs to 2A, 3Cpro, 3Dpro, or 3BVPg. These results were not cell-type specific or replication-dependent. They required only these particular proteins (2A or 3BCD) to be expressed within cells. During infection, however, the consequences of these nuclear activities were clearly advantageous to the virus. Deletions in 2A, which prevented nucleolar localization, were less effective at the shut off of cap-dependent host protein synthesis. Even more curious was the observation that nucleoli in Mengovirus-infected cells never seemed to shut off their synthesis (Fig. 2A). Nucleolar antibodies (e.g. B23 and C23), viral mAbs (2A), and fluorescein-UTP labels all showed progressively brighter foci throughout the infection and continuous rRNA synthesis, as if the pol-I and pol-III were stimulated into overdrive. Rhino- and polioviruses with different 2A proteins, and different host shut off mechanisms (cleavage of eIF-4G), do not act the same way in nucleoli (Amineva et al., submitted for publication). It is as if cardioviruses somehow require the upregulation of more than the usual cohort of ribosomes, and they encode 2A (plus 3BCD?) as the means of achieving this.

Another piece of the puzzle was the report by Vadim Agol, in a 1974 classic reference, that EMCV protein “G” (i.e. 2A) was generally found associated with ribosomes in infected cells (Medvedkina et al., 1974). Other more recent reports support these data, and suggest further that viral protein association with ribosomes may part of the host protein translational shut off pathway (Hensold et al., 1996). The antibodies introduced here have been able to confirm an association of 2A protein with ribosomes (results not shown),
and we are working to characterize this relationship more completely. One hypothesis to be explored is that cardioviruses may deliberately trigger an upregulation of rRNA synthesis so that 2A, localized to the nucleolus, can be "built-in" to new ribosomal subunits. When exported to the cytoplasm, these modified subunits would presumably have a strong 2A-directed preference for viral over host mRNA, effectively prevening host translation. Put simply, it is possible that the virus may induce the cell to make toxic ribosomes that only work on viral IRESs. Nefarious mechanism, is it not? If true, the hypothesis predicts that additional weaker (?) interactions between 2A and ribosomes might be responsible for the residual cytoplasmic inhibitory effects observed with the 2AASS protein.

But woven within this host-protein shut off scenario are other potential 2A activities. Host mRNA transcription directed by pol-II is also abrogated by cardiovirus infection, and clearly, there are massive changes in nuclear chromatin structure accompanying these shifts (Fig. 2E and Aminev et al., this special issue, 2003). Protein B23, which we now recognize as one likely binding partner for 2A, reacts strongly with core histones and is known to be involved in structural changes of chromatin, thereby regulating transcription and replication within the ribosomal DNA region. This protein also helps to maintain the nucleolar structure (Okuwaki et al., 2001; Chen and Huang, 2001). If 2A nuclear activities disrupted the normal B23 functions, there might be significant transcriptional consequences for pol-I, pol-III, and pol-II. We are in the process of developing novel 2A sequences that can selectively impinge on nuclear vs cytoplasmic pathways, so we can begin to tease apart these overlapping mechanisms.

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