Encephalomyocarditis Virus Leader Is Phosphorylated by CK2 and Syk as a Requirement for Subsequent Phosphorylation of Cellular Nucleoporins

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ABSTRACT

Encephalomyocarditis virus and *Theilovirus* are species in the *Cardiovirus* genus of the *Picornaviridae* family. For all cardioviruses, the viral polyprotein is initiated with a short Leader (L) protein unique to this genus. The nuclear magnetic resonance (NMR) structure of L_E from encephalomyocarditis virus (EMCV) has been determined. The protein has an NH₂-proximal CHCC zinc finger, a central linker, and a contiguous, highly acidic motif. The theiloviruses encode the same domains, with one or two additional, COOH-proximal domains, characteristic of the human Saffold viruses (SafV) and Theiler's murine encephalomyelitis viruses (TMEV), respectively. The expression of a cardiovirus L, in recombinant form, or during infection/transfection, triggers an extensive, cell-dependent, antihost phosphorylation cascade, targeting nucleoporins (Nups) that form the hydrophobic core of nuclear pore complexes (NPC). The consequent inhibition of active nucleocytoplasmic trafficking is potent and prevents the host from mounting an effective antiviral response. For this inhibition, the L proteins themselves must be phosphorylated. In cells (extracts or recombinant form), L_E was shown to be phosphorylated at Thr₄₇ and Tyr₄₁. The first reaction (Thr₄₇), catalyzed by casein kinase 2 (CK2), is an obligatory precedent to the second event (Tyr₄₁), catalyzed by spleen tyrosine kinase (Syk). Site mutations in L_E, or kinase-specific inhibitors, prevented L_E phosphorylation and subsequent Nup phosphorylation. Parallel experiments with L_S (SafV-2) and L_T (TMEV BeAn) proteins confirmed the general cardiovirus requirement for L phosphorylation, but CK2 was not the culpable kinase. It is likely that L_S and L_T are both activated by alternative kinases in different cell types, probably reactive within the Theilo-specific domains.

IMPORTANCE

An understanding of the diverse methods used by viruses to interfere with cellular processes is important because they can teach us how to control virus infections. This report shows how viruses in the same genus use different cellular enzymes to phosphorylate their proteins. If these processes are interfered with, the viruses are severely disabled.

Cardiovirus in the *Picornaviridae* family are subdivided into two species, the encephalomyocarditis viruses (EMCV) and theiloviruses (Theiler's murine encephalitis virus [TMEV]). EMCV-R is the prototype isolate for this genus. Although various EMCV have been isolated from diverse mammalian hosts, predominantly these are rodent viruses. Most are fairly homogeneous in sequence, host range, and pathogenicity (for a review, see reference 1), in that they cause myocarditis, encephalitis, or diabetes-like symptoms (2). The TMEV, in contrast, include variants such as Vilyuisk human encephalomyelitis virus, Theiler's-like rat virus, Theiler's murine encephalitis virus (TMEV), and Saffold virus 1 (SafV-1) to SafV-8. Strains of TMEV are subdivided into those which are neurovirulent (e.g., GDVII and FA) and those that cause persistent demyelination (e.g., DA and BeAn). The demyelinating strains are frequently used to model multiple sclerosis etiologies in mice (for a review, see reference 3). The SafV are human viruses (4). They are yet to be linked to any particular human disease, although recent work suggests that they can persist in their hosts (5) and that SafV-2 has the potential to become neurotropic when inoculated intracerebrally into mice (6).

A hallmark of all cardioviruses is the short Leader (L) protein translated at the NH₂ terminus of the viral polyprotein (see Fig. S1 in the supplemental material). While other picornaviruses (e.g., *Aphthovirus* and *Erbovirus* L^{Pro}) can encode alternative proteins at this location, the cardiovirus Ls always display an unusual CHCC

zinc-binding domain, a central linker, a short acidic region, and additional Ser/Thr-rich motifs, characteristic of their species (7–11). After translation, these highly charged (pI of ~3.7) proteins are released from an L-P1-2A precursor by the activities of the downstream 3C protease. After release during infection, or as a recombinant protein, the L from EMCV-R (L_E; 67 amino acids [aa]) triggers a cellular phosphorylation cascade aimed at the nucleoporins (Nups), which form the hydrophobic core of nuclear pore complexes (NPC). The degree of Nup hyperphosphorylation is so extensive, and so unlike anything that happens during the normal cell cycle, that it completely disrupts all active host nucleocytoplasmic trafficking pathways (12–14) (J. Ciomperlik, unpublished results). The altered NPC become open to widespread passive diffusion, allowing essential components to leak from the nucleus and become available for cytoplasmic viral rep-

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lication. At the same time, active trafficking of signaling proteins (e.g., for interferon induction) or transport of transcribed cellular mRNAs ceases abruptly. This potent, unique antihost response presumably prevents the cell from mounting an effective retaliation against the virus and is probably the reason most EMCV are highly pathogenic to mice (50% lethal dose [LD₅₀] = <100 PFU) (15).

The L_E-dependent Nup phosphorylation cascade requires an early obligate binding between L_E and the NPC transport regulatory protein, Ran-GTPase (16, 17). Stoichiometric interaction requires catalytic facilitation by RCC1, a nuclear-localized guanine-nucleotide exchange factor (18). Once bound, L_E:Ran complexes are virtually nondissociable. They have a K_D (equilibrium dissociation constant) of ~3 nM and cannot be disrupted by exogenous nucleotides, implying that Ran becomes locked into some virus-preferred format (19). L_E:Ran is the foundation complex through which host kinases are subsequently recruited in poorly understood secondary steps to carry out the actual Nup hyperphosphorylation events. Inhibition of extracellular signal-regulated kinase 1 (ERK1)/ERK2 or p38 pathways prevents L_E activities during EMCV-R infection, identifying these kinases as participants if not as the actual phosphate contributors (20).

Complicating this picture are observations (10, 21) and predictions (7, 22) that L_E, as well as L_T (from TMEV), is itself phosphorylated during infection. L_E exposed to eukaryotic cells or cytosol reacts with antibodies (Ab) specific to phosphotyrosine, predicted as Tyr₄₁ (10), but other studies suggest that Thr₄₇ is the target site (21). Mutation here (Thr₄₇Ala) disrupts L_E functions during infection, such that active NPC trafficking inhibition is delayed (13), Nup phosphorylation is abrogated (12), and alpha/beta interferon (IFN-α/β) transcription is no longer blocked (23). The L_E functions can be partially restored by substituting a phosphomimetic glutamate (Thr₄₇Glu) at this key position (12, 13, 23). However, *in vitro* Nup phosphorylation assays with digitonin-treated cells and recombinant L_E are not responsive to mutation at Tyr₄₁ or Thr₄₇ (10, 14). Casein kinase II (CK2) can phosphorylate recombinant L_E at Thr₄₇ in cell-free assays (21), but there is no evidence this is the kinase that acts upon L_E *in vivo*; neither is there evidence in cells, or in any recombinant reconstructions, that currently discriminates L_E, L_T, or L_S (SafV) phosphorylation as a consequence or antecedent to the Nup phosphorylation cascades.

The L_T and L_S proteins share several properties with L_E, including the NH₂ zinc-binding domain, central linker, acidic domain, and a short COOH terminal motif that includes the 3C^{pro} cleavage site (see Fig. S1 in the supplemental material). They are shorter by 7 residues at the immediate NH₂ terminus but instead have insertions of 10 (SafV) or 15 (TMEV) extra residues just upstream of the COOH motif. The extra common residues ("Theilo domain"), and the extra TMEV-specific residues (serine/threonine [Ser/Thr] domain), make L_S (71 aa) and L_T (76 aa) longer than the L_E, although they all are expected to have reasonably similar biological functions (11). The L_T (DA strain) has been linked to disruption in active NPC trafficking (24), induction of Nup phosphorylation (specifically Nup98) (25), and inhibition of interferon responses (26). The L_E from EMCV-R can replace L_T (DA) in recombinant virus, albeit with reduced viral replication efficiency (11). Neither L_T nor L_S from any strain has ever been tested directly as a phosphorylation substrate. Thr₆₃ of L_T (DA) was suggested as such, because Thr₆₃Ala substitution reduced Nup98 phosphorylation in L929 cells. Virus with this mutation had re-

duced toxicity to BALB/3T3 cells, while an analogous phosphomimetic, Thr₆₃Asp, retained the wild-type phenotype (7). The L_T Ser₅₇ (DA, BeAn) locale was also proposed as a putative phosphorylation site, because as one of the few known sequence discontinuities in the Ser/Thr-rich domain, this amino acid (Pro₅₇, GDVII) correlates with virus growth kinetics in BHK cells (22).

Now, with recombinant proteins, infections, transfections, and cell-free assays, we have identified 2 sequential L_E phosphorylation steps. Neither influences the initial L_E binding to Ran (17), and yet both are both required for subsequent activation of the L-dependent Nup hyperphosphorylation pathways. The primary event at Thr₄₇ is catalyzed by cellular CK2. The secondary event at Tyr₄₁ is catalyzed by cellular spleen tyrosine kinase (Syk). In parallel, recombinant L_T (BeAn) and L_S (SafV-2) are shown to be directly phosphorylated by various cell extracts, including HeLa, but CK2 is not the primary agent, and the sites are not orthologous to L_E.

MATERIALS AND METHODS

Plasmids. Bacterial plasmids encoding active N-terminal glutathione S-transferase (GST)-tagged EMCV-R L protein (GST-L_E) have been previously described (9, 16). The EMCV-R sequence (GenBank M81861) was from infectious cDNA pEC₉ (15). Pilot studies indicated that analogous GST fusions were not active for L_T and L_S proteins (not shown), so a new panel was engineered to place equivalent GST tags at the COOH termini, using multistep PCR and a common backbone vector (pTriEx1.1; Novagen). A GST amplicon from pDEST24 (Invitrogen) converted the normal initiation AUG into a Leu codon (see Table S1 in the supplemental material; primers 1437 and 1390). Ligation into the backbone vector (pTriEx1.1) preceded ligation with a second amplicon (primers 1426 and 1345) encoding L_E from pFluc/L (16). The vector sequence upstream of the L_E AUG codon was then altered to provide stronger translation activity during eukaryotic transfection (primers 1433 and 1434). Plasmids pL_T-GST and pL_S-GST were constructed in parallel. A SafV-2 L amplicon was generously provided by Howard Lipton, after PCR from a full-length cDNA (primers 1238 and 1239). After subcloning, the L_S gene was reamplified (primers 1393 and 1394) and then ligated into the backbone vector (pL_S-GST). Plasmid pL_T-GST was similar, except the starting cDNA was from TMEV (BeAn), using primers 1120 and 1121 (into pGEX-6p-2; Amersham Biosciences). Two more steps into pT1.1 (primers 1244 and 1245) and then into pT1.1-GST (primers 1391 and 1392) completed the process. The members of the final plasmid set (pL_E-GST, pL_S-GST, and pL_T-GST) differ only in the sequence of the L genes. Site-directed mutagenesis used 2-step PCR on these templates (27) with the indicated inside (mutagenic) primers and flanking primers (see Table S1 in the supplemental material) to create single- or double-mutant sequences. Sequencing and restriction digests confirmed all constructs. The panel encodes T7 and cytomegalovirus (CMV) promoters and readily expressed the respective L_X-GST proteins as COOH-terminal GST fusions in *Escherichia coli* (after IPTG [isopropyl-β-D-thiogalactopyranoside] induction) or in HeLa cells after plasmid transfection.

Protein expression and isolation. Protein samples for GST-L_E, L_E-GST, L_T-GST, and L_S-GST and mutated derivatives were prepared as described previously (9, 17). Briefly, after IPTG induction of plasmid-transformed *E. coli* (Rosetta competent cells; Novagen), the lysates were fractionated with glutathione Sepharose high-performance columns (GE Healthcare Life Sciences). Retained protein was exchanged into buffer (10 mM Bis-Tris propane, 50 mM NaCl, 2 mM dithiothreitol [DTT], pH 7.4) via spin column concentration (Millipore) and then subjected to anion-exchange chromatography using a Bio-Scale Mini Ion Exchange cartridge (Bio-Rad). The L_X-GST peaks were concentrated (into 25 mM HEPES–150 mM KCl–2 mM DTT, pH 7.3) and stored at –80°C. Protein samples prepared this way typically contain 10% to 20% GST truncation products from premature termination within the open reading frame (ORF), in

addition to the full-length Lx-GST. In contrast, GST-L_E preparations, especially those which undergo an additional Sephacryl S-100 fractionation (17), do not have such fragments. His-Ran was expressed and purified as described previously (17). Recombinant kinases CK2 (New England BioLabs), Syk (SignalChem), and Src (Cell Sciences) were obtained commercially.

Cell procedures. HeLa cells (ATCC CRL-1958) were grown in suspension (37°C, 10% calf serum, 5% CO₂) in modified Eagle's medium. At 24 h preinfection or transfection, they were plated into dishes. Infection (multiplicity of infection [MOI] = 30) with vEC₉ (15) used an attachment period (30 min, 20°C) before the cells were transferred to 37°C (4 to 5 h). In kinase inhibition studies, the cells were pretreated with TBCA [(E)-3-(2,3,4,5-tetrabromophenyl) acrylic acid] (Calbiochem) (10 μM) or TBB (4,5,6,7-tetrabromo-2-azabenzimidazole) (Calbiochem) (50 μM) for 1 h preinfection. The samples were maintained in the dark before, during, and after (4 to 5 h) infection. Transfections with pLx-GST cDNAs (1 μg) used Lipofectamine (Invitrogen) (1 μg) techniques (37°C, 5% CO₂, overnight) in 24-well plates with Opti-MEM media (Invitrogen). BHK-21 cells were a generous gift from John Yin. At harvest, all cells were washed with phosphate-buffered saline (PBS), lysed in gel loading buffer (SDS), boiled, and then fractionated by Laemmli SDS-PAGE.

Western analyses. Proteins fractionated by SDS-PAGE were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) and then blocked (10% nonfat dry milk) in Tris-buffered saline (TBST; 20 mM Tris [pH 7.6], 150 mM NaCl, 0.5% Tween 20) (20°C, 1 h). The membranes were washed (3×) with TBST before incubation (4°C, overnight) with an appropriate primary antibody (1% milk-TBST). The antibodies included αNup (monoclonal Ab 414 [MAB414]; Covance) against Phe/Gly-containing Nups, αGST (MAB 71087; Novagen), and αtubulin (MAB T4026; Sigma-Aldrich). After further washes (3× TBST), a secondary antibody (α-mouse A2554; Sigma-Aldrich) was added (1% milk-TBST), incubated (20°C, 1 h), and then removed (3×; TBST) before the membranes were reacted with enhanced chemiluminescence substrate (GE Healthcare) and exposed to film.

Phosphorylation reactions. HeLa or BHK-21 cytosol from uninfected cells was prepared via Dounce homogenization (28). Rabbit reticulocyte lysates were commercial (Promega). For inhibitor testing, TBCA (10 μM)-dimethyl sulfoxide (DMSO) or TBB (50 μM)-DMSO was employed to pretreat (30 min, 37°C, in the dark) the HeLa cytosol before use, and then the TBCA-DMSO and TBB-DMSO were maintained in the reaction mixtures at the same concentrations. Phosphorylation reaction mixtures (80 μl, with 30 μl cytosol, 2 μl 10 mM ATP, 2 μg Lx-GST, 0.75 μl [γ-³²P]ATP, and ~12 μCi/μl) were incubated in GST binding buffer (50 mM HEPES, 150 mM NaCl, 0.5% NP-40, pH 7.4) for 45 min (37°C). Glutathione Sepharose 4B beads (GE Healthcare Life Sciences) (10 μl per sample) were then added, followed by agitation (room temperature, 2 h). The beads were collected by centrifugation (500 × g), washed with GST buffer (4×), and then boiled with SDS gel loading buffer. Protein fractionation was by SDS-PAGE (8% to 10%), with band visualization by phosphor imaging (Typhoon 9200 Variable Mode Imager; GE Healthcare), silver staining, or Western analysis.

Reactions performed with recombinant kinases were similar, except that CK2 (New England BioLabs) (10 U), Syk (SignalChem) (10.3 U), and/or Src (Cell Sciences) (10 U) replaced the cytosol and the commercial buffers recommended for these enzymes were used. Phosphorylation reactions performed with GST-L_E:Ran complexes were initiated with GST- or GST-L_E-bound Ran complexes on glutathione Sepharose 4B beads, as described previously (17). The beads were collected by centrifugation (500 × g) and then resuspended in reaction buffer (commercial for each kinase; 25 μl) containing 0.5 μl [γ-³²P] ATP (12.16 μCi/μl), CK2 (10 U), Syk (10.3 U), or both enzymes (10 U each). Incubation was performed at 37°C for up to 60 min. For Syk reaction quantitation, L_E-GST was preincubated with CK2 and cold ATP before addition of Syk and [γ-³²P]ATP. The bead-bound complexes were washed, boiled with SDS, and fractionated by SDS-PAGE.

Phosphorylation quantitation. After Western assays or phosphorimaging, the gel-fractionated proteins bands were scanned by densitometry (TL 100 [TotalLab]; Sigma-Aldrich). Observed pixels were normalized to GST or tubulin loading controls in the wild-type (L_E) or untreated (inhibitor) control samples. Stoichiometry measurements required the excision of (³²P) gel bands after autoradiography (4 independent lanes per sample type), followed by scintillation counting, using a method similar to that described by Hollinger and Hepler (29). Controls and calibrated standards measured the degree of quench (4% to 8%). The dpm of recovered ionizing radiation was divided by the [γ-³²P]ATP-specific activity (304 μCi/μM) to determine the recovered phosphate. The protein content of analogous samples was assayed with bicinchoninic acid (BCA) protein assay reagent kits (Thermo Scientific). Typically, this averaged ~78 pM of GST or GST-L_E per isolated gel slice. Stoichiometry, expressed as percent, recorded recovered phosphate (pM) per recovered protein (pM). Values > 100% indicate multiple phosphorylation events (29).

Sequence analysis. A data set of unique cardiomyocyte L sequences (see Fig. S1 in the supplemental material) was compiled from GenBank and aligned by ClustalX (30). Posttranslational modification predictions were performed with NetPhos2.0 (31), Phosphomotif Finder (32), and Phosida (33). Alignments were displayed with Weblogo (34).

RESULTS

L_E mutagenesis. Recombinant EMCV (Mengo strain) Leader protein (L_M) can be phosphorylated *in vitro* with CK2 at Thr₄₇ (21). Deletion analysis and ³²P-labeling experiments performed by Dvorak et al. (10) showed that the closely related EMCV-R L protein (L_E) is phosphorylated during infection at a Tyr residue, presumed to be Tyr₄₁. Motif identification algorithms NetPhos 2.0 (31), Phosida (33), and PhosphoMotif Finder (32) concurred that both sites could be kinase targets (see Fig. S1 in the supplemental material), but it was unknown whether these sites were alternatives or additive. Accordingly, single- and double-substitution mutations (Tyr₄₁Phe, Thr₄₇Ala) were engineered into recombinant L_E-GST cDNA and the isolated proteins were tested for ³²P incorporation (γ-ATP) after incubation with HeLa cell extracts (Fig. 1A). GST tags were included because the small, highly charged L_E proteins by themselves are captured inefficiently by membranes during blotting steps (10, 14, 16). Relative to the wild-type protein, Thr₄₇Ala and Tyr₄₁Phe reduced label incorporation by 82% and 40%, respectively. It required both mutations to eliminate all detectable phosphorylation. Parallel, single-proton nuclear magnetic resonance (NMR) determinations confirmed that these L_E sequences were properly folded (17). Therefore, both sites are phosphorylation targets and they contribute additively.

Phosphorylation by CK2 and Syk. Kinase identification by motif prediction is an uncertain process because many enzymes accept numerous related substrates, and there is often functional redundancy. Of the multiple algorithms queried in this study, all concurred that L_E Thr₄₇ fit the general consensus, [S/T]XX[D,E], for CK2. The Tyr₄₁ site was anticipated less frequently, but when identified, spleen tyrosine kinase (Syk) and the related Src tyrosine kinase (Src) were the most commonly suggested enzymes. Syk and Src both phosphorylate a family of overlapping substrates, but neither has a precisely defined target consensus with which they are guaranteed to react. Generically, both accept Tyr in a regional acidic environment (+3, -3), with a preference for nearby aliphatics (35, 36).

When (commercial) recombinant CK2 was added to GST-L_E, the reactions reached completion within 10 to 15 min (Fig. 2B). Samples (4×) from each time point were measured for both protein content and ³²P incorporation, allowing an approximation of

FIG 1 L_x-GST phosphorylation in cytosol. (A) Recombinant L_E-GST and its phosphorylation site mutants were reacted with HeLa cytosol and [γ -³²P]ATP. After SDS-PAGE, bands were detected by phosphor imaging (³²P) or Western analyses (α GST). Captured pixels were normalized relative to the wild type (wt) for ³²P and GST signals (Norm). (B) As described for panel A, the proteins in the recombinant L_x-GST panel were reacted with cytosol from HeLa cells, BHK cells, or rabbit reticulocytes (Rabbit retics). Normalization was to L_E-GST.

the stoichiometry. Although there is always variation in such measurements (see Table S2 in the supplemental material), in this case, the values fluctuated around a median of ~96% (standard deviation [StDev], $\pm 37\%$), or close to saturation. Surprisingly, parallel reactions with Syk had virtually undetectable incorporation when the enzyme was tested alone (Fig. 2A). But when both enzymes were added together (Fig. 2C), the ³²P counts more than doubled over that of CK2 alone, to a median of ~195% (StDev, $\pm 57\%$). In this case, completion required 15 to 30 min of reaction. These results are consistent with a secondary, Syk-dependent phosphorylation of GST-L_E at Tyr₄₁, sequential to that of CK2 at Thr₄₇. Reactions with Src instead of Syk were not equivalent (Fig. 2D).

This enzyme did not react with GST-L_E even when added in combination with CK2.

During infection, L_E undergoes a tight 1:1 binding interaction with Ran-GTPase as an obligate step in active NPC trafficking inhibition (16, 19). The complexes form between GST-L_E and Ran recombinant proteins (17) in the complete absence of L_E phosphorylation. The hinge-linker contact points for L_E (Lys₃₅-Trp₄₀) lie immediately adjacent to the Tyr₄₁ and Thr₄₇ phosphorylation sites, and although neither mutation inhibits complex formation (17), it was unknown whether Ran binding subsequently masked these sites or whether L_E phosphorylation needed to occur before Ran binding. Preformed recombinant GST-L_E:Ran complexes for the panel of L_E phosphorylation mutants were reacted with CK2 or Syk or a combination of these kinases. The phosphomimetic (Thr₄₇Asp) was included as a structural mimic for pseudophosphorylation at this site. As with the isolated GST-L_E samples, CK2 readily labeled the wild-type protein as well as the Tyr₄₁Phe mutant, the only other sequence with an intact Thr₄₇ (Fig. 3A). When similar complexes were prereacted with CK2 (cold ATP) and then labeled in the presence of Syk, the wild type and the phosphomi-

FIG 2 Phosphorylation quantitation. (A, B, and C) GST-L_E was reacted with recombinant Syk (A), CK2 (B), or Syk plus CK2 (C) for the indicated times (min). After SDS-PAGE, samples were cut from the gel(s) and analyzed for protein and ³²P content (see Materials and Methods). Averaged values for 4 replicates (see Table S2 in the supplemental material) are shown as “%Phos.” (D) As described for panel B and C, GST-L_E was reacted with recombinant Src or with Src plus CK2. (E) As described for panel B, the panel of L_x-GST proteins was reacted with CK2. In this case, captured pixels were normalized to L_E-GST for ³²P and GST signals (Norm).

FIG 3 Phosphorylation of GST-L_E:Ran complexes. GST-L_E and the indicated mutant derivatives were reacted with recombinant His-Ran. The stoichiometric (1:1) complexes were isolated as described previously (17). Equivalent fractions were reacted with [γ -³²P]ATP and CK2 (A), CK2 plus Syk (C), or Syk (B) as described for Fig. 2 (60 min). The Syk-only reactions were preceded with a prior incubation with CK2 and cold ATP. After SDS-PAGE, bands were detected by phosphorimaging (³²P) or silver stain (“S-stain”). Captured pixels were averaged ($n = 4$) and normalized relative to the wild type (wt) for ³²P signals (Norm). For panels A and B, “100” denotes the maximum signal for one phosphorylation event. For panel C, “100” denotes the maximum signal for up to two events.

FIG 4 CK2 inhibitors. (A) HeLa cytosol was pretreated with TBCA or TBB before L_E -GST was added with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described for Fig. 1A. (B) GST- L_E was reacted with recombinant CK2 in the presence of TBCA or TBB and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described for Fig. 2B (45 min). After SDS-PAGE, bands were detected by phosphorimaging (^{32}P) or silver staining (S-stain). Captured pixels were normalized relative to the wild type (wt) for ^{32}P signals (Norm).

metic were the only active substrates (Fig. 3B). Simultaneous addition of CK2 and Syk again allowed Tyr₄₁Phe to be actively labeled (Fig. 3C). Clearly, neither kinase was inhibited by the Ran binding status of GST- L_E . With or without Ran, CK2 reactions at Thr₄₇ or phosphomimetic reactions at this position were obligatory precursors to subsequent Syk reactions at Tyr₄₁.

CK2 inhibition. HeLa cell extracts contain endogenous CK2, and presumably this enzyme is responsible for initial L_E -GST reactions in that context (e.g., see Fig. 1A). The addition of CK2 inhibitors TBCA and TBB reduced HeLa cytosol-mediated incorporation significantly (Fig. 4A). However, neither drug was completely effective, with 57% and 61% inhibition, respectively. Parallel reactions with recombinant CK2 in buffer gave similar results (Fig. 4B), indicating that these particular inhibitors do not prevent all activity by this enzyme. The experimental “normalized” values in the cytosol were determined by relative densitometry and cannot distinguish L_E -GST samples with 1 or 2 added phosphates. Even partial phosphorylation by the primary kinase could (putatively) have still allowed reactions with the secondary enzyme. Collectively, though, the data demonstrate that CK2 is present in HeLa extracts and, logically, that it acts as the natural, dominant kinase for the first L_E event (at Thr₄₇), just like the purified enzyme.

L_E -GST and Nup phosphorylation. Digitonin-treated HeLa cells in the presence of *Xenopus* or HeLa extracts undergo GST- L_E -dependent hyperphosphorylation of Nups (14). The activity can be assayed by upward mobility shifts, particularly for Nup₆₂, when samples are labeled with ^{32}P or reacted with Nup-specific antibodies. GST- L_E with large deletions (e.g., removal of the acidic domain), or with gross structural rearrangements (e.g., C₁₉A), do not induce this activity. Surprisingly, recombinant L_E with single point mutations at Tyr₄₁ and Thr₄₇ are functional in this assay, although mutation to Thr₄₇ in mengovirus is debilitating to virus growth (10, 14, 21). A key difference in these approaches is the dose of Leader protein. Digitonin assays expose nuclei to high levels of GST- L_E . Infections provide significantly lower concentrations of translated L_E . To test the Leader phosphorylation muta-

FIG 5 Nup phosphorylation after transfection. HeLa cells were transfected with the panel of L_E -GST cDNAs (see Materials and Methods). After 24 h, the cells were collected, lysed, and then fractionated by SDS-PAGE. Western analyses were performed with αNup and $\alpha\text{tubulin}$. Phosphoshift was quantified using TotalLab Quant. Normalization was relative to tubulin and the wild-type (wt) signal. Upward mobility shift values were quantified as described by Porter et al. (20). (B) As described for panel A, cDNAs for L_X -GST and inactive (C_XA) mutant derivatives were transfected into HeLa cells and then monitored for Nup₆₂ phosphorylation activity.

tions independently of infection, the panel of matched L_X -GST cDNAs was transfected into HeLa cells. As expected, the C₁₉A conformational mutation prevented Nup₆₂ phosphorylation (Fig. 5A). The Thr₄₇Ala mutation and the phosphomimetic mutation, Thr₄₇Asp, both reduced the Nup₆₂ phosphorylation signal to 33% to 34%. The observed values at this primary site were lower than for the secondary Tyr₄₁Phe mutation (by itself). However, when both GST- L_E phosphorylation sites (Tyr₄₁Phe plus Thr₄₇Ala) were changed, there was no observable shift in the Nup₆₂ band. Therefore, at least one and preferably both L_E phosphorylation events are prerequisites for this activity.

The Leader phosphorylation requirement is also manifest during virus infection. When vEC₉ is introduced into HeLa cells, Nups become phosphorylated in a L_E -dependent manner (14). But exposure of the cells with TBCA or TBB reduced the Nup₆₂ phosphor shifts to 62% and 16%, respectively (Fig. 6). For intact cells, TBCA is specific for CK2 at this concentration (Calbiochem

FIG 6 Nup phosphorylation during infection. HeLa cells were pretreated with TBCA or TBB and then infected with vEC₉ (MOI = 30). The inhibitors were maintained during infection. Harvested cells (5 h) were fractionated by SDS-PAGE and subjected to Western analyses as described for Fig. 5.

screen of 28 common kinases), as is TBB (Calbiochem screen of 33 common kinases). CK2 itself is not responsible for Nup phosphorylation (data not shown and reference 20). The process occurs only when there is properly folded L_E that can act as a phosphorylation-competent substrate for CK2 and Syk.

Other cardioviruses. The TMEV and SafV Leader proteins are orthologs and presumed functional analogs to L_E with regard to Nup phosphorylation triggers. Well-characterized cDNAs for SafV-2 and TMEV (BeAn) were chosen to represent this species. L_S and L_T share 43% and 39% amino acid identity relative to L_E , respectively (see Fig. S1 in the supplemental material). Matched L_S -GST and L_T -GST genes were cloned into the same expression vector as L_E -GST. Upon transfection of HeLa cells, both theilovirus proteins induced Nup₆₂ phosphorylation, but neither was as effective as GST- L_E , where the band shift was nearly twice as pronounced (Fig. 5B). All the shifts were L_X -dependent activities, though, because disruption of each Leader conformation with a debilitating zinc-finger mutation (i.e., Cys₁₁Ala) significantly reduced Nup₆₂ phosphorylation.

Among the known SafV and TMEV viruses, none conserve the identical context of Thr₄₇ required for primary kinase recognition of L_E (see Fig. S1 in the supplemental material). That theilovirus Leader proteins are themselves phosphorylated *in vitro* or *in vivo*, or whether such activity is dependent on cell type, has never been demonstrated. Transfections (e.g., L_X -GST) and infections rarely produce enough Leader from any cardiovirus for a thorough study of native phosphorylation status. Instead, as a first test of such activity, the matched set of recombinant L_X -GST proteins were reacted with cytosol from HeLa cells, BHK cells, or rabbit reticulocytes (Fig. 1B). All 3 extracts labeled L_E -GST and L_T -GST with ³²P, albeit with different efficiencies. For L_E -GST, the preferred efficiency, normalized to the amount of protein, was reticulocytes > BHK > HeLa, with a nearly 4-fold difference over the spectrum of cell types. For L_T -GST, it was reticulocytes > HeLa > BHK. Equivalent L_S -GST was labeled only weakly in HeLa and BHK and not phosphorylated at all in reticulocyte extracts.

The various reactivities of these proteins in multiple cell types would be expected if each Leader sequence relied on different kinases for self-phosphorylation. Indeed, when recombinant CK2 was added to these proteins, it was unable to label L_S -GST or L_T -GST (Fig. 2E). Therefore, while phosphorylation is obviously part of the Leader activity profile for all cardioviruses, each species (or clade?) seems to take advantage of its unique sequences to present preferred sites to different endogenous kinases, perhaps in a cell-specific manner.

DISCUSSION

Viral proteins can be phosphorylated by cellular kinases for a variety of functional reasons. The dengue virus NS5 RNA-dependent RNA polymerase, for example, has a bipartite nuclear localization signal (NLS) that is turned on and off by phosphorylation with CK2 (37). In fact, CK2 is one of the most commonly identified kinases utilized by viruses to phosphorylate their proteins (38). Before the current report, it was known that the EMCV Leader protein was phosphorylated during infection, presumably by CK2 at Thr₄₇. The significance of that modification, or of dual modification at Thr₄₇ and Tyr₄₁, was unclear.

The data here show that both L_E phosphorylations, first at Thr₄₇ and then at Tyr₄₁, are required to optimize the ability of

EMCV to trigger Nup phosphorylation and consequently inhibit active cellular nucleocytoplasmic trafficking. Logically, such an important, initial reactive step should be performed by a constitutively active, ubiquitous kinase such as CK2 (39). L_E mutations and CK2 inhibitors identified this enzyme in cells or recombinant form as reactive with Thr₄₇. The event was an obligate precursor to subsequent phosphorylation with Syk at Tyr₄₁. Although several other kinases, for example, glycogen synthase kinase, are known to require CK2 activity before they can act (40, 41), to our knowledge this is the first example of CK2 priming a Syk event. Syk, another ubiquitous kinase, usually selects tyrosine sites surrounded by negative charges (42). Yet Tyr₄₁ within the highly acidic domain of L_E (pI 3.7) was completely unreactive unless CK2 was added beforehand. Cooperation between Syk and CK2 has been reported before. Luz et al. found that CK2 and Syk cophosphorylate CFTR, the protein responsible for cystic fibrosis. Those data attributed the primary phosphorylation event to Syk, with the consequence as altered CFTR trafficking (43).

The exact cellular mechanistic steps enabled by L_E phosphorylation remain to be determined, although intracellular localization or changes in binding partner preference are certainly possibilities. Syk reactions at Tyr₄₁ were able to bypass the requirement for prior phosphorylation by CK2 at Thr₄₇ when a phosphomimetic Asp was encoded at this position (Fig. 3). However, both sites needed to be wild type and properly phosphorylated for maximum Nup phosphorylation (Fig. 5). Surprisingly, the L_E phosphorylation status does not contribute to Ran binding (19) and the protein could be readily labeled after complex formation (Fig. 3). If simple L_E trafficking were affected by phosphorylation, one might not expect this result. Rather, we favor the idea these L_E modifications play a subsequent role, perhaps in the selection or retention of the actual Nup phosphorylation agents, such as ERK1/2 and p38 (20; J. Ciomperlik, unpublished results). Now that sequential phosphorylations by CK2 and Syk are acknowledged precursors to L_E -directed active trafficking inhibition, the next steps in reconstruction the full reaction cascade can be approached.

The theilovirus Leaders L_T and L_S are presumed to have analogous functions to L_E , and yet they differ in the display of 1 to 2 short peptide insertions that are exceptionally rich in putative cellular phosphorylation motifs. Neither the L_T with [C₄₀]XDLD nor the L_S with [T₄₀]XXL[D,E] from any described virus in this species has the required CK2 consensus sequence (see Fig. S1 in the supplemental material). Furthermore, only the L_S but not the L_T sequences maintain the potential secondary site (Tyr₃₄) equivalent to L_E Tyr₄₁. CK2 does not react with either protein in recombinant form, although both proteins do become phosphorylated when treated with extracts from HeLa or BHK cells (Fig. 1). It will require extensive mutagenesis, NMR folding studies, and “kinase guesses” to establish where and how L_T and L_S encode their alternative sites. That they behave differently from each other in reticulocyte extracts suggests that the individual theilovirus domains may be targeted independently. It is clear, however, that each of these proteins participates directly in the pathways causing Nup phosphorylation. Transfection of L_X -GST cDNA into cells directly triggered this activity. Parallel L_X zinc-finger mutations were inhibitory in all cases (Fig. 5). These functional differences are conceivably clade-specific adaptations to particular cell types, and the respective differential phosphorylation patterns are most probably the key to the variability of these proteins.

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REFERENCES

- Carocci M, Bakkali-Kassimi L. 2012. The encephalomyocarditis virus. *Virulence* 3:351–367. <http://dx.doi.org/10.4161/viru.20573>.
- Yoon JW, Onodera T, Notkins AL. 1977. Virus-induced diabetes mellitus: VIII. Passage of encephalomyocarditis virus and severity of diabetes in susceptible and resistant strains of mice. *J. Gen. Virol.* 37:225–232.
- Oleszak EL, Chang JR, Friedman H, Katsetos CD, Platsoucas CD. 2004. Theiler's virus infection: a model from multiple sclerosis. *Clin. Microbiol. Rev.* 17:174–207. <http://dx.doi.org/10.1128/CMR.17.1.174-207.2004>.
- Jones MS, Lukashov VV, Ganac RD, Schnurr DP. 2007. Discovery of a novel human picornavirus in a stool sample from a pediatric patient presenting with fever of unknown origin. *J. Clin. Microbiol.* 45:2144–2150. <http://dx.doi.org/10.1128/JCM.00174-07>.
- Himeda T, Hosomi T, Okuwa T, Muraki Y, Ohara Y. 2013. Saffold virus type 3 (SAFV-3) persists in HeLa cells. *PLoS One* 8:e53194. <http://dx.doi.org/10.1371/journal.pone.0053194>.
- Hertzler S, Liang Z, Trespo B, Lipton HL. 2011. Adaptation of Saffold virus 2 for high-titer growth in mammalian cells. *J. Virol.* 85:7411–7418. <http://dx.doi.org/10.1128/JVI.00265-11>.
- Ricour C, Borghese F, Sorgeloso F, Hato SV, van Kuppeveld FJ, Michiels T. 2009. Random mutagenesis defines a domain of Theiler's virus leader protein that is essential for antagonism of nucleocytoplasmic trafficking and cytokine gene expression. *J. Virol.* 83:11223–11232. <http://dx.doi.org/10.1128/JVI.00829-09>.
- Chen H-H, Kong W-P, Roos RP. 1995. The leader peptide of Theiler's murine encephalomyelitis virus is a zinc-binding protein. *J. Virol.* 69:8076–8078.
- Cornilescu CC, Porter FW, Zhao Q, Palmenberg AC, Markley JL. 2008. NMR structure of the Mengovirus leader protein zinc-finger domain. *FEBS Lett.* 582:896–900. <http://dx.doi.org/10.1016/j.febslet.2008.02.023>.
- Dvorak CMT, Hall DJ, Hill M, Riddle M, Pranter A, Dillman J, Deibel M, Palmenberg AC. 2001. Leader protein of encephalomyocarditis virus binds zinc, is phosphorylated during viral infection and affects the efficiency of genome translation. *Virology* 290:261–271. <http://dx.doi.org/10.1006/viro.2001.1193>.
- Paul S, Michiels T. 2006. Cardiovirus leader proteins are functionally interchangeable and have evolved to adapt to virus replication fitness. *J. Gen. Virol.* 87:1237–1246. <http://dx.doi.org/10.1099/vir.0.81642-0>.
- Bardina MV, Lidsky P, Sheval EV, Fominykh KV, van Kuppeveld FJ, Polyakov VY, Agol V. 2009. Mengovirus-induced rearrangements of the nuclear pore complex: hijacking cellular phosphorylation machinery. *J. Virol.* 83:3150–3161. <http://dx.doi.org/10.1128/JVI.01456-08>.
- Lidsky PL, Hato S, Bardina MV, Aminev AG, Palmenberg AC, Sheval EV, Polyakov VY, van Kuppeveld FJ, Agol V. 2006. Nucleo-cytoplasmic traffic disorder induced by cardioviruses. *J. Virol.* 80:2705–2717. <http://dx.doi.org/10.1128/JVI.80.6.2705-2717.2006>.
- Porter FW, Palmenberg AC. 2009. Leader-induced phosphorylation of nucleoporins correlates with nuclear trafficking inhibition of cardioviruses. *J. Virol.* 83:1941–1951. <http://dx.doi.org/10.1128/JVI.01752-08>.
- Hahn H, Palmenberg AC. 1995. Encephalomyocarditis viruses with short poly(C) tracts are more virulent than their Mengo virus counterparts. *J. Virol.* 69:2697–2699.
- Porter FW, Bochkov YA, Albee AJ, Wiese C, Palmenberg AC. 2006. A picornavirus protein interacts with Ran-GTPase and disrupts nucleocytoplasmic transport. *Proc. Natl. Acad. Sci. U. S. A.* 103:12417–12422. <http://dx.doi.org/10.1073/pnas.0605375103>.
- Bacot-Davis VR, Palmenberg AC. 2013. Encephalomyocarditis virus Leader protein hinge domain is responsible for interactions with Ran GTPase. *Virology* 443:177–185. <http://dx.doi.org/10.1016/j.virol.2013.05.002>.
- Klebe C, Prinz H, Wittinghofer A, Goody RS. 1995. The kinetic mechanism of Ran-nucleotide exchange catalyzed by RCC1. *Biochemistry* 34:12543–12552. <http://dx.doi.org/10.1021/bi00039a008>.
- Petty RV, Palmenberg AC. 2013. Guanine-nucleotide exchange factor RCC1 facilitates a tight binding between EMCV Leader and cellular Ran GTPase. *J. Virol.* 87:6517–6520. <http://dx.doi.org/10.1128/JVI.02493-12>.
- Porter FW, Brown B, Palmenberg A. 2010. Nucleoporin phosphorylation triggered by the encephalomyocarditis virus leader protein is mediated by mitogen-activated protein kinases. *J. Virol.* 84:12538–12548. <http://dx.doi.org/10.1128/JVI.01484-09>.
- Zoll J, Melchers WJ, Galama JM, van Kuppeveld FJ. 2002. The mengovirus leader protein suppresses alpha/beta interferon production by inhibition of the iron/ferritin-mediated activation of NF-kappa B. *J. Virol.* 76:9664–9672. <http://dx.doi.org/10.1128/JVI.76.19.9664-9672.2002>.
- Takano-Maruyama M, Ohara Y, Asakura K, Okuwa T. 2006. Theiler's murine encephalomyelitis virus leader protein amino acid residue 57 regulates subgroup-specific virus growth on BHK-21 cells. *J. Virol.* 80:12025–12031. <http://dx.doi.org/10.1128/JVI.00693-06>.
- Hato SV, Ricour C, Schulte BM, Lanke KH, de Bruijn M, Zoll J, Melchers WJ, Michiels T, van Kuppeveld FJ. 2007. The mengovirus leader protein blocks interferon-alpha/beta gene transcription and inhibits activation of interferon regulatory factor 3. *Cell. Microbiol.* 9:2921–2930. <http://dx.doi.org/10.1111/j.1462-5822.2007.01006.x>.
- Delhay S, van Pesch V, Michiels T. 2004. The leader protein of Theiler's virus interferes with nucleocytoplasmic trafficking of cellular proteins. *J. Virol.* 78:4357–4362. <http://dx.doi.org/10.1128/JVI.78.8.4357-4362.2004>.
- Ricour C, Delhay S, Hato SV, Olenyik TD, Michel B, van Kuppeveld FJ, Gustin KE, Michiels T. 2009. Inhibition of mRNA export and dimerization of interferon regulatory factor 3 by Theiler's virus leader protein. *J. Gen. Virol.* 90:177–186. <http://dx.doi.org/10.1099/vir.0.005678-0>.
- van Pesch V, van Eyll O, Michiels T. 2001. The leader protein of Theiler's virus inhibits immediate-early alpha/beta interferon production. *J. Virol.* 75:7811–7817. <http://dx.doi.org/10.1128/JVI.75.17.7811-7817.2001>.
- Ho SH, Hunt HD, Horton RM, Pullen JK, Pease LR. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59. [http://dx.doi.org/10.1016/0378-1119\(89\)90358-2](http://dx.doi.org/10.1016/0378-1119(89)90358-2).
- Watters K, Palmenberg A. 2011. Differential processing of nuclear pore complex proteins by rhinovirus 2A proteases from different species and serotypes. *J. Virol.* 85:10874–10883. <http://dx.doi.org/10.1128/JVI.00718-11>.
- Hollinger S, Hepler JR. 2004. Methods for measuring RGS protein phosphorylation by G protein-regulated kinases. *Methods Mol. Biol.* 237:205–219.
- Larkin M, Blackshields G, Brown N, Chenna R, McGettigan P, McWilliam H, Valentin F, Wallace I, Wilm A, Lopez R, Thompson J, Gibson T, Higgins D. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948. <http://dx.doi.org/10.1093/bioinformatics/btm404>.
- Blom N, Gammeltoft S, Brunak S. 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* 294:1351–1362. <http://dx.doi.org/10.1006/jmbi.1999.3310>.
- Amanchy R, Periaswamy B, Mathivanan S, Reddy R, Tattikota SG, Pandey A. 2007. A curated compendium of phosphorylation motifs. *Nat. Biotechnol.* 25:285–286. <http://dx.doi.org/10.1038/nbt0307-285>.
- Gnad F, Gunawardena J, Mann M. 2011. PHOSIDA 2011: the posttranslational modification database. *Nucleic Acids Res.* 39:D253–D260. <http://dx.doi.org/10.1093/nar/gkq1159>.
- Crooks GE, Hon G, Chandonia J-M, Brenner SE. 2004. WebLogo: a sequence logo generator. *Genome Res.* 14:1188–1190. <http://dx.doi.org/10.1101/gr.849004>.
- Xue L, Wang W-H, Iliuk A, Hu L, Galan J, Yu S, Hans M, Geahlen RL, Tao WA. 2012. Sensitive kinase assay linked with phosphoproteomics for identifying direct kinase substrates. *Proc. Natl. Acad. Sci. U. S. A.* 109:5615–5620. <http://dx.doi.org/10.1073/pnas.1119418109>.
- Heizmann B, Reth M, Infantino S. 2010. Syk is a dual-specificity kinase that self-regulates the signal output from the B-cell antigen receptor. *Proc. Natl. Acad. Sci. U. S. A.* 107:18563–18568. <http://dx.doi.org/10.1073/pnas.1009048107>.
- Forwood JK, Brooks A, Briggs LJ, Xiao CY, Jans DA, Vasudevan SG. 1999. The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site. *Biochem. Biophys. Res. Commun.* 257:731–737. <http://dx.doi.org/10.1006/bbrc.1999.0370>.
- Keating J, Striker R. 2012. Phosphorylation events during viral infections provide potential therapeutic targets. *Rev. Med. Virol.* 22:166–181. <http://dx.doi.org/10.1002/rmv.722>.
- Grankowski N, Boldyreff B, Issinger OG. 1991. Isolation and characterization of recombinant human casein kinase II subunits alpha and beta from bacteria. *Eur. J. Biochem.* 198:25–30. <http://dx.doi.org/10.1111/j.1432-1033.1991.tb15982.x>.

40. Wang Y, Roach PJ. 1993. Inactivation of rabbit muscle glycogen synthase by glycogen synthase kinase-3. Dominant role of the phosphorylation of Ser-640 (site-3a). *J. Biol. Chem.* **268**:23876–23880.
41. Fiol CJ, Wang A, Roeske RW, Roach PJ. 1990. Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates. *J. Biol. Chem.* **265**:6061–6065.
42. Hornbeck P, Kornhauser J, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M. 2012. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res.* **40**:D261–D270. <http://dx.doi.org/10.1093/nar/gkr1122>.
43. Luz S, Kongsuphol P, Mendes A, Romeiras F, Sousa M, Schreiber R, Matos P, Jordan P, Mehta A, Amaral M, Kunzelmann K, Farinha C. 2011. Contribution of casein kinase 2 and spleen tyrosine kinase to CFTR trafficking and protein kinase A-induced activity. *Mol. Cell. Biol.* **31**: 4392–4404. <http://dx.doi.org/10.1128/MCB.05517-11>.