Cardiovirus Leader proteins bind exportins: Implications for virus replication and nucleocytoplasmic trafficking inhibition

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Cardiovirus Leader proteins (Lx) inhibit cellular nucleocytoplasmic trafficking by directing host kinases to phosphorylate Phe/Gly-containing nuclear pore proteins (Nups). Resolution of the Mengovirus Lm structure bound to Ran GTPase, suggested this complex would further recruit specific exportins (karyopherins), which in turn mediate kinase selection. Pull-down experiments and recombinant complex reconstitution now confirm that Crm1 and CAS exportins form stable dimeric complexes with enchaphalomyocarditis virus Lx, and also larger complexes with Lx:Ran. shRNA knockdown studies support this idea. Similar activities could be demonstrated for recombinant Lx and Lt from Theiloviruses. When mutations were introduced to alter the Lx zinc finger domain, acidic domain, or dual phosphorylation sites, there was reduced exportin selection. These regions are not involved in Ran interactions, so the Ran and Crm1 binding sites on Lx must be non-overlapping. The involvement of exportins in this mechanism is important to viral replication and the observation of trafficking inhibition by Lx.

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Introduction

Eukaryotic nuclei have double-layered membranes studded with an estimated 2800 channels called nuclear pore complexes (NPC). Each pore is comprised of about 30 nucleoporin proteins (Nups) arranged with octagonal symmetry (Terry and Wente, 2009). Many of these Nups, especially those lining the central channel, are hydrophobic, displaying characteristic Phe/Gly-repeat units which serve as a physical barrier to casual diffusion of molecules >40 kDa. Larger macromolecules cannot, by themselves, navigate the disordered, hydrophobic Nup tangle that constitutes the central channel. Instead, directionally targeted cargos display short nuclear localization signal motifs (NLS) or nuclear export signal motifs (NES) that are recognized and bound by karyopherin receptors (importins or exportins) traveling in the required direction. The cargo/receptor selection process is regulated by a Ran GTPase-moderated energy gradient, which determines the active, or inactive status of the various karyopherins (Nakielny and Dreyfuss, 1999).

There are multiple virus types which usurp these transport pathways to enhance their own replication or prevent activation of innate responses. Cardioviruses in the Picornaviridae family use the unique strategy of induced hyper-phosphorylation directed at multiple Phe/Gly Nups, to bring about a rapid, virtually complete inhibition of cellular import/export processes (Porter et al., 2006). The effect is toxic to cells, but allows these cytoplasmic viruses to replicate with apparent impunity. Encephalomyocarditis virus (EMCV), Mengovirus, Theiler’s murine encephalomyelitis virus (TMEV) and Saffold virus (Saf) are characteristic isolates which have been studied for these activities (Porter and Palmenberg, 2009; Basta and Palmenberg, 2014).

Each Cardiovirus encodes a small, highly-charged Leader protein (Lx, 67–76 amino acids) at the N-terminus of its polyprotein, which, when introduced into cells is sufficient to recapitulate the entire Nup hyper-phosphorylation phenomenon (Porter et al., 2006). Solution structures of the Mengo protein (Lm) show a CHCC amino-proximal zinc finger domain as the dominant motif in an otherwise flexible conformation (Bacot-Davis et al., 2014; Corniellescu et al., 2008). A central “hinge” region and C-proximal acidic region assume more defined induced-fit conformations only when Lm interacts with Ran GTPase, a strong (3 nM) preferred binding target when tested with recombinant proteins, or as detected with native proteins within the infected cells’ nuclear rims (Porter et al., 2006; Bacot-Davis et al., 2014; Petty and Palmenberg, 2013). The complex locks Ran into an irreversible “active” structure, nearly identical (–1.6 Å RMSD) to nucleotide-bound RanGTP (Bacot-Davis et al., 2014). In uninfected cells, this conformer of Ran would normally chaperone interactions between export
karyopherins and their cargos, and then participate in shuttling these complexes through the NPC from nucleus to cytoplasm. But since Ran and Lx are not kinases, it is clear that neither can be directly responsible for catalyzing infection-dependent Nup phosphorylation. Instead, it has been proposed that Lx:Ran complexes nucleate subsequent (irreversible?) reactions with exportins, which in turn then carry the culpable activated kinase cargos directly to Nup targets (Bacot-Davis et al., 2014). In recent support of this model, recombinant LE-GST (EMCV), when reacted with HeLa cytosol was shown to pulldown immunologically detectable amounts of Crm1 (chromosome region maintenance 1 protein, XPO1) and CAS (cellular apoptosis susceptibility protein, XPO2) exportins. The responsible kinases, as implicated with parallel inhibition studies, are known to include MAPK agents from the ERK1 and p38 pathways (Porter et al., 2010). Presumably, these enzymes, for which the activated forms have dominant nuclear localizations (Roux and Blenis, 2004), are somehow preferentially selected for Nup phosphorylation activities by the Lx:Ran:exportin complexes.

Other than the Lx:Ran interactions, though, few of these steps have been broadly explored experimentally. Moreover, complicating the overall picture is an additional mechanistic requirement for dual Lx phosphorylation. Ran interactions with Lx do not require these modifications, but exportin extraction by recombinant Lx (Bacot-Davis et al., 2014), and ultimately Nup phosphorylation (Basta and Palmenberg, 2014; Basta et al., 2014), are sensitive to the motif status. Cardiovirus Lx proteins vary somewhat in the sequence and lengths of their hinge and acidic regions, and it is within this variable context that the required phosphorylation residues occur. We now report new results showing direct, reciprocal binding interactions between native and recombinant Lx proteins, with cellular and recombinant exportins, Crm1 and CAS. The data refine the existing mechanistic model and moreover suggest that these specific interactions may be mediated by the Lx zinc finger, acidic domains and phosphorylation sites, which do not directly participate in Ran binding.

Results

Screen for Lx binding partners

Ran GTPase, as a binding partner for GST–Lx, was previously identified by reciprocal pull-down activities and confirmed with Western analyses using anticipated reactive antibodies (Porter et al., 2006; Bacot-Davis et al., 2014). Related experiments suggested the phosphorylation status of Lx was a key determinant for additional relevant interactions, particularly with exportins (Bacot-Davis et al., 2014). As a broader screen for dominant cytosolic partners, HeLa lysates were pre-cleared with glutathione beads and then reacted with GST–Lx in the presence of added ATP. These conditions allow efficient dual phosphorylation of Lx (Basta et al., 2014) while reducing background binding by non-specific proteins. After SDS-PAGE fractionation, 7 prominent band regions (Fig. 1) were excised, pooled, digested with trypsin and submitted for orbi-trap mass spectrometry. Table 1 summarizes the strongest sequence hits as defined by positive identification of at least 30 unique peptides each. The list includes exportin 1 (Crm1) and exportin 2 (CAS), the nuclear transport-associated karyopherins which had been modeled previously with probable Lx:Ran interactions (Bacot-Davis et al., 2014). The sizes of these proteins, 123 kDa and 110 kDa, respectively, are consistent with the principal band 2 included in pool A. Therefore, Lx, as it is phosphorylated in this cytoplasmic context, does indeed react strongly with exportins. Interestingly, 6 of the 9 strongest hits (Cover%) covering both pools identified several other proteins with structurally analogous, HEAT repeat motifs, similar to those characteristic of the exportins, perhaps indicating a topological class of preferred contact interactions with Lx.

Mutational mapping (Bacot-Davis and Palmenberg, 2013) and determination of the solution structure of Lm bound to Ran GTPase (Bacot-Davis et al., 2014) explored sequence contributions from the central hinge region (aa 35–40), the zinc finger (aa 14–20), the acidic domain (aa 37–59) and phosphorylation sites (Tyr41, Thr47) as important to the observation of Lx-dependent Nup phosphorylation. However, Cys19Ala (zinc finger), 4D4A (acidic domain) and Tyr41Ala/Thr47Ala phosphorylation changes did not significantly affect Lx:Ran interactions with endogenous or recombinant proteins. Those contacts are mediated primarily by the Lx hinge region, including W40 (Bacot-Davis and Palmenberg, 2013). With regard to exportins though, a subset panel of GST-tagged Lx proteins with same sequence changes (3D3A not 4D4A) were all less efficient than unmodified protein (wt) at extracting endogenous Crm1 and CAS from HeLa cell lysates (Fig. 2A). Relative to the acidic domain deletion (ΔA) a negative control, which misfolds the remainder of the protein (Bacot-Davis and Palmenberg, 2013), the 4 tested substitutions in the other crucial Nup phosphorylation areas reduced exportin extraction by 50–90%. Exportin extraction was not unique to the Lx sequences. Equivalent experiments with Lx (Saf) and Lm (TMEV) recombinant proteins showed these materials too, reacted readily (Fig. 2B). In a reverse challenge, antibodies to Crm1, when attached to beads, were similarly able to find both intended target (native Crm1) and GST–Lx spiked into the lysates (Fig. 2C). Again, the most efficient conditions for these pulldowns required the full Lx sequence. Mutational mapping and structure determinations place the W40 site primarily within the GST–Lm:Ran interface, although NMR suggests there is considerable conformational motion (Bacot-Davis et al., 2014; Bacot-Davis et al., 2014).
and Palmenberg, 2013). The inefficiency with which Crm1 (baits) is extracted by αCrm1 (compare Fig. 2C mock to wt) and the stoichiometric excess (> 100 × ) of GST–LE make this particular experiment somewhat less sensitive to changes in binding efficiencies, relative to Fig. 2A, particularly with regard to this specific mutation.

**Direct interaction with Crm1**

HeLa lysates contain endogenous Ran GTase. Moreover, these lysate-based systems will efficiently phosphorylate the first site and partially phosphorylate the second site of any added cardiovirus Leader (LX) proteins displaying intact sequences (Basta and Palmenberg, 2014). But with cell-free GST–LE systems, the phosphorylation status can be precisely controlled by sequential addition of the relevant kinases. Syk activity at Tyr41 requires pre-phosphorylation with AmpK or CK2 (compare Fig. 2C mock to wt) and the observation of complexes near the void volume, were

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*Fig. 1* gel bands 1,2,6,7 were pool A, bands 3,4,5 were pool B.

*Fig. 2A* mock to wt)

ultimately, Nup phosphorylation itself is the test for mechanistic involvement in LX–dependent activities. Crm1 is an important protein with multiple cellular roles (Hutten and Kehlenbach, 2006), especially during mitosis. Its functions are not entirely compensated by alternative exportins like CAS (reviewed in Wu et al. (2013)). Nonetheless, HeLa cells transduced with an shRNA against Crm1 remained viable, divided and survived without visible cytopathic effect for more than a week after doxycycline induction. Under such conditions, within the first 2 days, ~60% of the Crm1 protein was turned over (Fig. 5A). By day 3, this value increased to ~80%. Induction alone did not allow Nup phosphorylation, but if the cells were also infected with vEC9 (attenuated, recombinant EMCV), the diagnostic upward smear of Nup62 was observed within 4 h PI. If the infection were delayed until 2, 3 or 4 days post-induction (PK) there was correspondingly lower levels of Nup phosphorylation and the virus replicated more poorly. This was evidenced both by the lower levels (normalized to tubulin per sample) of viral polymerase proteins 3D and 3 CD (Fig. 5A), and by reductions in progeny titer, as determined by plaque assay (Fig. 5C).

**Complex formation**

When the Fig. 3A experiment was repeated with stepwise addition of the components, again, GST–LE (1 added phosphate) extracted rCrm1 regardless of whether rRan (±RCC1) was added before (“a”), or after (“b”) as the tertiary component. As a preliminary estimate of whether these 3 known proteins could collectively interact in vitro, dual phosphorylated GST–LE, rRan and rCrm1 were mixed, incubated and then fractionated over a Superdex 200 column. The absorbance values (Fig. 4B) and parallel silver-stained gels (Fig. 4C) placed about 20% of the signals from all 3 proteins within early eluates, near the trailing edge of the void volume (fractions 35–40). The column sensitivity in this region is too low to assign specific stoichiometries, but trimeric associations (~190 kDa) and/or larger combinations of GST–LE:αCrm1:rRan would be expected to elute here. There were additional strong peaks consistent with GST–LE:αCrm1 complexes (fractions 67–75) and also monomers of rRan (fractions 85–90). It cannot be excluded that some material at the front edge of the late-eluting rRan peak also contains Leader material (GST–LE:rRan) that was carried below the limits detectable by silver-stain. In any case, the central fractions show conclusively that rCrm1 (120 kDa) and phosphorylated GST–LE (35 kDa) do readily interact in apparent 1:1 stoichiometry. Similar to the pulldown experiments (Fig. 3B), parallel chromatographs confirmed that the intensity of this peak and the observation of complexes near the void volume, were dependent on the phosphorylation status GST–LE (not shown).

**shRNA knockdown of Crm1**

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with reduced levels of Crm1. This phenomenon is directly attributable to the LX mechanism reliance on Crm1, because when similar shRNA-induced cells were transfected (4 days PK) with cDNAs for the LE, Lx or Lx proteins, Nup62 phosphorylation was again reduced (Fig. 5B).

Crm1 inhibition

Leptomycin B (LMB) is a well-studied fungal antibiotic (Hamamoto et al., 1983) which alkylates most iterations of eukaryotic Crm1 proteins. It binds covalently to Cys528, effectively alkylating most iterations of Crm1 inhibition again reduced (Fig. 5B).

Fig. 2. GST-tagged Lx mutations. Bait proteins, GST–LE and related mutant derivatives (A) as well as LE–GST, Lx–GST and Lx–GST (B) were incubated with HeLa cytosol. After glutathione sepharose bead extractions, Western analyses determined the amount of bound Crm1 and CAS, relative to the wt samples (% intensity); (C) GST–LE and mutant derivatives (prey) were mixed with HeLa cytosol (+0.5 μM ATP) then incubated with protein-G sepharose beads linked to αCrm1 (bait). After boiling and protein fractionation, the Crm1 and GST signals were detected by Western analyses. In these blots, the VH and VL coupling antibody proteins were also reactive with the secondary antibodies. (*) indicates LE–GST mutant derivatives.

Fig. 3A. None of the rRan and rCrm1 pulldowns were obviously altered by the presence of this drug. Infected cells, monitored for Nup62 phosphorylation over a range of vEC9 MOI, gave similar results (Fig. 6B) in that the presence of the drug was essentially ineffective. Such cells gave equivalent progeny titers regardless of whether LMB was added (Fig. 6C). The GST–LE recognition of Crm1, then, must be outside of this binding site, or can be compensated for by CAS activities.

Discussion

In cells or recombinant assays, cardiovirus Leader proteins react tightly with Ran GTPase, forcing this nuclear trafficking regulator into a conformation indistinguishable from the “active” GTP-bound format required for subsequent exportin binding (Bacot-Davis et al., 2014). Formation of this complex is absolutely required for subsequent exportin binding (Bacot-Davis et al., 2014). Resolution of the LM:Ran structure confirmed the central hinge region of Lmø (e.g. Trp40), tucked under the C-tail of Ran, forms the key interaction domain. The Lmø zinc finger (e.g. C19), phosphorylation sites (Y141, T27), and the majority of C-proximal acidic domain (e.g. Asp148, Asp151, Asp152) do not contribute to these (singly phosphorylated GST–LE) behaved the same as they did in Fig. 3A.
contacts, even though each of these regions has proven essential to the observation of L<sub>E</sub>-dependent Nup phosphorylation (Porter et al., 2006; Bacot-Davis and Palmenberg, 2013). The resolved structure predicts a model whereby these non-Ran binding segments might instead mediate subsequent interactions with the karyopherin class of exportins (e.g. Crm1, CAS), which require exactly this GTP-like Ran conformation for nuclear entry (Bacot-Davis et al., 2014). Validation of this idea, that L<sub>E</sub> and exportins can directly interact, was the rationale for the experiments described here.

Native cardiovirus L<sub>E</sub> proteins are refractive to Western analyses and antibody induction because of their small size and high charge. Recombinant GST-tagged L<sub>E</sub> materials readily recapitulate lyses and antibody induction because of their small size and high charge. Recombinant GST-tagged L<sub>E</sub> (CK2 adds 1 phosphate) was pre-incubated with stoichiometric amounts of Crm1 and rRan (+ RCC1) for lanes labeled with “a”. Then, rCrm1 and rRan (+ RCC1) were added to previously deficient samples (“b”) before a second incubation period. Glutathione bead extraction and Western visualization was as in Fig. 3. (B) Dual phosphorylated GST-L<sub>E</sub> (CK2 plus Syk) was incubated with Crm1 and rRan (+ RCC1). The sample was fractionated on a size exclusion column pre-calibrated with MW markers. Fraction absorbance aligned with (C) SDS-PAGE (silver stain) identified eluted complexes. Bands labeled “rCrm1<sup>+</sup>” are minor bacterial contaminants from the rCrm1 purification procedure.

The current mechanism for L<sub>E</sub>-dependent Nup hyperphosphorylation expects that within the nuclear rim, L<sub>E</sub>:Crm1 complexes select exportins carrying activated kinases such as ERK1 and p38, and it is this multiplex which tethers within the nuclear pores and phosphorylates the Nups (Bacot-Davis et al., 2014). Interestingly, we could not define a required sequence of addition for rRan and Crm1 to a mutual GST–L<sub>E</sub> complex with the current reactions. Both proteins appeared to interact independently, especially if L<sub>E</sub> was properly phosphorylated. Near the void volume of the fractionation column, about 20% of the added mass eluted with signals from all 3 proteins. Whether these are the expected trimers or other high-mass complexes is not clear at present. Three additional anomalies from this experiment, the significant peak of free Ran, the dominance of GST–L<sub>E</sub>:Crm1, and the lack of rRan:rCrm1 complexes, suggest that we do not yet understand all the interplay among these proteins. In cells, especially within the context of nuclear pores, the cooperative, active formats of L<sub>E</sub>, Ran and Crm1 (or CAS) are sure to be influenced by the local environment, especially the GNP nucleotide gradient and perhaps other cellular (catalytic?) proteins.

In cell-free reactions, a catalytic amount of RCC1 significantly accelerates binding saturation (Pett and Palmenberg, 2013), locking L<sub>E</sub> and Ran into (virtually) in-dissociable complexes which mimic the topology of RanGTP even in the absence of a nucleotide (Bacot-Davis et al., 2014). The same RanGTP format is required for Ran:Crm1 (or CAS) interactions (Guttler et al., 2010; Guttler and Gorlich, 2011). From our experiments, it is now clear that GST–L<sub>E</sub>, preferably with at least 1 phosphate attached, can interact strongly and independently with both rRan and rCrm1 (or CAS). Void-volume complexes were observed with all three proteins, but we don’t yet know how to drive these assemblages to saturation. Possibly, the RCC1-dependent Ran morphing, an aid for good GST–L<sub>E</sub> interactions, works contrary to simultaneous rRan:rCrm1 cellular protein was found to block the other’s extraction, nor was extraction of either or both apparently sensitive to the sequence of addition (Figs. 3, 4 and 6). Such results were not entirely surprising, because the structure model predicts that the L<sub>E</sub> zinc finger, acidic domain and phosphorylation sites, which do not make Ran contacts, are exactly the segments which should mediate putative exportin contacts (Bacot-Davis et al., 2014). In other words, the binding domains are non-overlapping. In support of this idea, mutations in GST–L<sub>E</sub> affecting any of the 3 of the predicted contact regions significantly diminished the extraction of native and recombinant Crm1. The reduced affinity was particularly apparent when the L<sub>E</sub> phosphorylation sites (Tyr<sub>41</sub>, Thr<sub>47</sub>) were mutated, or when GST–L<sub>E</sub> in vitro, was restricted in its phosphorylation status. Dual phosphorylated GST–L<sub>E</sub>, reacted with rCrm1, fractionated by gel filtration at the size expected for a stoichiometric complex. An additional finding in favor of the current model is that L<sub>E</sub> interaction with Crm1 apparently did not involve the NES cargo recognition site (HEAT repeats H11 and H12) inhibited by LMB. Within fully assembled Crm1:L<sub>E</sub>:Ran complexes, the Nup phosphorylation kinases (activated ERK and p38 pathway kinases) would presumably need to bind here, or nearby, as preferred export cargos (Bacot-Davis et al., 2014).

Clearly, exportins, particularly Crm1 and CAS, are strong viable partners for L<sub>E</sub>. An inducible Crm1 knockout cell line was created to link the requirements for Crm1 to EMCV replication. As was shown previously for the Ran mechanistic requirement (Porter et al., 2006), Nup62 phosphorylation and viral fecundity, as measured by 3D/3CD synthesis and by progeny titers, were strongly dependent upon the pool of native Crm1. This was also true when induced cells were transfected with cDNAs from any 3 cardiovirus L<sub>E</sub> proteins, indicating a common requirement for Crm1, if not other analogous exportins, in the collective Nup phosphorylation pathways.
binding by stripping endogenous nucleotides from the added Ran. This situation would explain the observed pool of free Ran, unable to dislodge GST–LE which is already tied up in rCrm1 complexes. It would also explain the lack of an observable rRan:rCrm1 peak, independent of the presence of GST–LE, as this should have been a strong native interaction, and indeed a driving force in normal nuclear trafficking. If true, then within the context of real nuclear pores, the innate nucleotide gradient must play a significant role in ternary complex assembly efficiency.

Materials and methods

Recombinant proteins

Plasmids encoding GST–LE, GST–LEΔA, GST–LEW40A, and related derivatives are described (Cornilescu et al., 2008; Petty and Palmenberg, 2013; Bacot-Davis and Palmenberg, 2013), as are analogous Lx–GST proteins from Saffold 2 and Theiler’s virus (BeAn), LS–GST, LT–GST, LE–GST and LE–GST Thr41Ala/Tyr47Ala (Basta and Palmenberg, 2014; Ciomperlik et al., 2015). LE–GST 3D3A was made similarly by introducing point mutations to alter the 3 codons for Asp48, Asp51 and Asp52 to Ala. For recombinant proteins, these cDNAs were transformed into BL21 Escherichia coli. The cells were incubated in media (0.5% glucose, 20μM ZnCl2, 50 mg/mL ampicillin, 30°C, 3 h) before induction with IPTG (1 mM) and harvest (6 h). Cells were re-suspended in GST buffer (50 mM Tris pH 7.4, 125 mM NaCl, 20 μM ZnCl2, 1 mM PMSF, 0.2 mM DTT, 0.5 mg/ml lysozyme), then sonicated. Debris was removed by centrifugation (20,000 x g, 45 min). The proteins were collected with GSTrap HP columns (GE Healthcare Bio-sciences, Pittsburgh PA) into modified GST buffer (10 mM glutathione
disulfide, 50 mM Tris, pH 8.0). Protein fractions were dialyzed into buffer QA (10 mM Bis-Tris Propane, 50 mM NaCl, 2 mM DTT, pH 7.4), then applied to a Macro-Prep High Q anion exchange column (BioRad, Hercules, CA). The fractions were eluted (buffer QA, 1 M NaCl) before dialysis (25 mM HEPES, 150 mM KCl, 2 mM DTT, pH 7.3), and assay for concentration and purity. A cDNA construct encoding amino-terminal, His-tagged murine Crm1 (rCrm1) was a kind gift from Dr. Dirk Görlich. Bacterial induction and protein isolation was as described (Guttler et al., 2010). Recombinant His-encoded amino-terminal, His-tagged murine Crm1 (rCrm1) was a kind gift from Dr. Dirk Görlich. Bacterial induction and protein isolation was as described (Guttler et al., 2010). Recombinant His-encoded amino-terminal, His-tagged murine Crm1 (rCrm1) was a kind gift from Dr. Dirk Görlich. Bacterial induction and protein isolation was as described (Guttler et al., 2010). Recombinant His-encoded amino-terminal, His-tagged murine Crm1 (rCrm1) was a kind gift from Dr. Dirk Görlich. Bacterial induction and protein isolation was as described (Guttler et al., 2010).  

Cell lysates

HeLa cells (ATCC CRL-1958) were grown in suspension (modified eagle’s medium, 10% calf serum, 5% CO2, 37 °C). Lysates were prepared after collecting the cells with centrifugation (500 g), washing the pellets (PBS), and resuspension (2 × volume) in hypotonic buffer (0.75 mM MgAc, 0.15 mM EGTA, 1 mM PMSF, 0.01 mg/ml leupeptin, 20 μM pepstatin A, 3 mM DTT). After a brief incubation (10 min, 4 °C) the samples were homogenized (dounce) or sonicated (2 × 30 s, 0 °C) before clarification (10 min, 16,000 g). The soluble fraction was supplemented with 1/10th volume 10 mM HEPES pH 7.3, 10 mM EGTA, 20 mM MgAc, 110 mM KAc, before aliquots were snap frozen and stored at −80 °C. Before use in pull-down experiments, the lysates were pre-reacted with glutathione sepharose beads and then re-clarified (as below).

Protein extraction

GST-tagged L₄ and mutant derivatives (bait, generally 2 μg) was incubated (37 °C, 45 min) with HeLa lysate (prey, 40 μl) before dilution (RIPA buffer, 50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1% IGEPA, pH 7.6), addition of glutathione sepharose beads (generally 10 μl per sample, GE Healthcare Bio-sciences) and agitation (3 h, 4 °C). When the pulldown prey was a recombinant protein, bait and prey were mixed stoichiometrically, diluted (to 100 μl), with TB, 200 mM HEPES pH 7.3, 1 mM EGTA, 2 mM MgAc, 110 mM KAc, before bead addition as above. Both types of samples were then further diluted (2 ×) with binding buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% IGEPA), and mixed end-over-end (1 h, 22 °C). For immunoprecipitation (IP), protein-G sepharose beads were conjugated to antibodies (bait, e.g. α-Crm1, 0.6 μl per 10 μl beads, overnight, 4 °C) before being rinsed, (1 × PBS, 0.02% Triton X-100), added to samples (prey, cytosol or recombinant protein, 10 μl/sample) and incubated (with rocking, 3 h, 4 °C). After pulldown or IP, the beads were rinsed (6 ×, 500 μl PBS, triton X-100, then 2 × 120 mM Tris, pH 6.8, 10% glycerol w/v, 2% SDS w/v, 200 mM DTT, 0.2% bromophenol blue) before the samples were denatured in a boiling water bath (10 min).

Mass spectroscopy

Protein bands extracted by GST–L₄ pulldown, were fractionated by SDS-PAGE and visualized by Coomassie staining before excision and digestion with trypsin according to protocols recommended by the UW-Madison Biotechnology Center. Briefly, gel samples were treated with extraction buffer 1 (200 μl, mM NH₄HCO₃, 50% acetonitrile (ACN), 45 min), and then dehydrated (100% ACN, 5 min) under vacuum. The samples were resuspended (40 μl, 40 mM NH₄HCO₃, 10% ACN) in the presence of trypsin gold (Promega, 20 pg/ml, plus 0.025% Protease Max surfactant, 1 h, 27 °C). Subsequently, extraction buffer 2 was added (200 μl, 40 mM NH₄HCO₃, 10% ACN) followed by incubation (overnight, 25 °C) and re-extraction (2 ×, 60 min, 50 μl 50% ACN, 5% trifluoroacetic acid). Resultant peptides from select bands (see Table 1) were pooled, dehydrated (2 h under vacuum) before purification and concentration via Ziptips (Millipore). The pool A and pool B samples were submitted for orbitrap mass spectroscopy at the UW-Madison Biotechnology Center.

Western analyses

Prepared samples were resolved by SDS-PAGE. The proteins were electro-transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were blocked (1 h, 10% NFD milk in TBST: 20 mM Tris pH 7.6, 150 mM NaCl, 0.5% Tween20) then incubated with a primary antibody (1% NFD milk in TBST, overnight, 4 °C) before washing (3 × TBST) and reaction with an appropriate secondary antibody (1 h, 20 °C). Antibodies included: αNup (murine mAb414, IgG, Covance, 1:2000), αtubulin (murine Ab, IgG, Sigma, 1:10,000), αGST (murine mAb, IgG, Novagen, 1:10,000), αCrm1 (rabbit Ab, IgG, Abcam, 1:2000), αCAS (goat Ab, IgG, Santa Cruz Biotechnology: 1:1000), αrN (goat Ab, IgG, Sigma, Santa Cruz Biotechnology: 1:1000), omouse (secondary Ab, IgG, Sigma Aldrich, 1:8000), αrabbit (secondary Ab, IgG, Promega, 1:8000), oggo (secondary Ab, IgG, Sigma, 1:8,000). For band visualization, the membranes were rinsed (3 ×, TBST), incubated (1 min) with enhanced chemiluminescence substrate (GE health-care) and then exposed to film. Densitometry analyses were as previously described (Basta et al., 2014).

Gel filtration

A HiLoad Superdex 200 16/600 chromatography column (GE Lifesciences) was calibrated with a commercial kit (Sigma-Aldrich MWGF1000-1kt) according to manufacturer’s instructions. Recombinant protein combinations were incubated (37 °C, 45 min), filtered (0.2 μm membrane) and volume adjusted (to 1 ml) before injection into the equilibrated column (50 mM HEPES, pH 7.4, 150 mM NaCl). After fractionation (0.2 ml/min, 4 °C) the collected samples (1 ml) were concentrated by dehydration under vacuum (0.5 ml) and assayed by SDS-PAGE, with silver stain, or Western techniques.

Cell procedures

HeLa cells plated to confluence, were infected with vEC9 (21) at an MOI of 1–20. This strain is a recombinant, short poly (C) attenuated EMCV. At 3–6 hours post-infection (37 °C, 5% CO₂) the cells were rinsed (2 ×, PBS) and collected into SDS buffer for protein analyses, or alternatively, subject to freeze-thaw (3 ×), lystate clarification and assays for plaque-forming units (Dvorak et al., 2001). Transfection assays used GST–L₄ cDNA (2.6 μg per 10⁶ cells in Lipofectamine 2000 (Life Technologies, Grand Island, NY)). After incubation (18 h, 37 °C, 5% CO₂) the cells were rinsed (2 ×, PBS) and harvested in SDS buffer. Leptomycin B (LMB, Sigma Aldrich, St. Louis, MO), was added as required (to 4 nM), by pretreating cells or buffers for 1 h. This concentration was then maintained throughout the experiment. A Crm1 knockout HeLa cell line was generated with the TRIPZ inducible lentiviral shRNA system (GE Dharmacon, shRNA VZTHS-172053) according to manufacturer instructions. The line was maintained under selection with puromycin. Plated cells were induced with doxycycline (0.25 nM, Sigma Aldrich). The media was refreshed daily (1–4 days) as required by the experiment.
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