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

Jessica J. Ciomperlik [a], Holly A. Basta [b], Ann C. Palmenberg [a,*]

[a] Institute for Molecular Virology and Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, United States
[b] Department of Biology, Rocky Mountain College, Billings, MT, United States

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ABSTRACT

Cardiovirus Leader proteins (L_X) inhibit cellular nucleocytoplasmic trafficking by directing host kinases to phosphorylate Phe/Gly-containing nuclear pore proteins (Nups). Resolution of the Mengovirus L_M 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 encephalomyocarditis virus L_E, and also larger complexes with L_E:Ran. shRNA knockdown studies support this idea. Similar activities could be demonstrated for recombinant L_S and L_T from Theiloviruses. When mutations were introduced to alter the L_E 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 L_E must be non-overlapping. The involvement of exportins in this mechanism is important to viral replication and the observation of trafficking inhibition by L_E.

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 (L_X, 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 (L_M) show a CHCC amino-proximal zinc finger domain as the dominant motif in an otherwise flexible conformation (Bacot-Davis et al., 2014; Cornilescu et al., 2008). A central “hinge” region and C-proximal acidic region assume more defined induced-fit conformations only when L_M 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 ($\sim$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

* Correspondence to: Institute for Molecular Virology, Robert M. Bock Laboratories, University of Wisconsin – Madison, 1525 Linden Drive, Madison, WI 53706, United States. Tel.: +1 608 262 7519; fax: +1 608 262 6690.
E-mail address: acpalmen@wisc.edu (A.C. Palmenberg).

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karyopherins and their cargos, and then participate in shuttling these complexes through the NPC from nucleus to cytoplasm. But since Ran and L_X are not kinases, it is clear that neither can be directly responsible for catalyzing infection-dependent Nup phosphorylation. Instead, it has been proposed that L_M: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 L_E-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 L_X:Ran:exportin complexes.

Other than the L_X:Ran interactions, though, few of these steps have been broadly explored experimentally. Moreover, complicating the overall picture is an additional mechanistic requirement for dual L_X phosphorylation. Ran interactions with L_M do not require these modifications, but exportin extraction by recombinant L_X (Bacot-Davis et al., 2014), and ultimately Nup phosphorylation (Basta and Palmenberg, 2014; Basta et al., 2014), are sensitive to the motif status. Cardiovirus L_X 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 L_X 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 L_X zinc finger, acidic domains and phosphorylation sites, which do not directly participate in Ran binding.

Results

Screen for L_E binding partners

Ran GTPase, as a binding partner for GST–L_E, 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 L_E 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–L_E in the presence of added ATP. These conditions allow efficient dual phosphorylation of L_E (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 L_E: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, L_E, 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

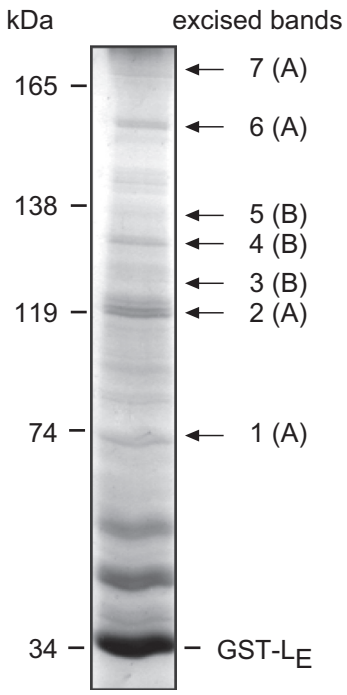

Fig. 1. Cytosol selection by GST–L_E. HeLa cytosol proteins (prey) reactive with GST–L_E (bait) were collected, gel fractionated and visualized with silver stain. Bands of interest (1–7) were excised, pooled (A or B), digested with trypsin and analyzed by orbi-trap mass spectroscopy for protein identity. kDa indicates the MW of proteins in a parallel marker lane.

analogous, HEAT repeat motifs, similar to those characteristic of the exportins, perhaps indicating a topological class of preferred contact interactions with L_E.

L_E association with exportins

Mutational mapping (Bacot-Davis and Palmenberg, 2013) and determination of the solution structure of L_M 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 (Tyr_{41}, Thr_{47}) as important to the observation of L_X-dependent Nup phosphorylation. However, Cys_{19}Ala (zinc finger), 4D4A (acidic domain) and Tyr_{41}Ala/Thr_{47}Ala phosphorylation changes did not significantly affect L_E:Ran interactions with endogenous or recombinant proteins. Those contacts are mediated primarily by the L_X hinge region, including W_{40} (Bacot-Davis and Palmenberg, 2013). With regard to exportins though, a subset panel of GST-tagged L_E 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 L_E sequences. Equivalent experiments with L_S (Saf) and L_T (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–L_E spiked into the lysates (Fig. 2C). Again, the most efficient conditions for these pulldowns required the full L_E sequence. Mutational mapping and structure determinations place the W_{40} site primarily within the GST–L_M:Ran interface, although NMR suggests there is considerable conformational motion (Bacot-Davis et al., 2014; Bacot-Davis