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The Lymphocytic Choriomeningitis Virus RING Protein Z Associates with Eukaryotic Initiation Factor 4E and Selectively Represses Translation in a RING-Dependent Manner

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Only a few host cell proteins that associate with arenaviruses have been identified. To date, the arenavirus Z protein associates with the promyelocytic leukemia protein PML and the ribosomal P proteins. The majority of PML is present in nuclear bodies which are translocated to the cytoplasm by infection with the arenavirus, lymphocytic choriomeningitis virus (LCMV). The Z protein is a small zinc-binding RING protein with an unknown function which is required for the viral life cycle. Here, we demonstrate an association between Z and the host cell translation factor, eukaryotic initiation factor 4E (eIF-4E) in infected and transfected cells. Z's association with both ribosomal proteins and this translation factor led us to investigate whether Z could modulate host cell translation. In cell culture, Z selectively represses protein production in an eIF-4E-dependent manner. Specifically, we see reduction in cyclin D1 protein production with no effect on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cells transfected with Z. Previous reports indicate that cyclin D1 is sensitive to eIF-4E levels, whereas GAPDH is not. Consistent with this, we observe preferential downregulation of cyclin D1 during infection and no effect on GAPDH. Further, no changes in RNA levels were observed for cyclin D1 or GAPDH transcripts. The interaction between eIF-4E and Z may provide a mechanism for slower growth observed in infected cells and a viral strategy for establishing chronic infection.

The prototype arenavirus, lymphocytic choriomeningitis virus (LCMV), is carried as an inapparent chronic infection of rodents, although primates can develop clinical signs similar to those of humans with Lassa fever (15, 16, 23). Arenaviruses such as LCMV and Lassa fever virus are noncytopathic in cell culture and easily establish chronic infection in their rodent hosts (23). The mechanism by which arenaviruses can maintain chronic infections in carrier hosts is not well understood. Arenaviruses have two single-stranded RNA genome segments and no introns and do not produce DNA intermediates during genome replication (23). Arenaviruses encode five products: a nucleocapsid protein (NP), an envelope glycoprotein (GP) that is processed into GP1 and GP2, an RNA polymerase (L), and an 11-kDa protein containing a RING finger domain (Z). The RING motif is ~60 residues in length, binds two zinc atoms, and is involved in mediating protein protein interactions (5, 24).

The Z protein function is unknown. It is packaged into both LCMV and Tacaribe virions, suggesting that it may function immediately after infection (8, 22). Biochemical studies of the Z protein from Tacaribe virus suggest a role for Z in genome synthesis. The Z proteins are highly conserved amongst the arenaviridae (7). Expression of Z in uninfected cells can reduce cell survival in serum starved fibroblasts, and this activity is partially mediated through its RING domain (3). Other viral proteins temper the actions of Z since LCMV-infected cells survive serum starvation better than their uninfected counterparts (3).

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LCMV associates with the promyelocytic leukemia protein PML during infection (1). Interestingly, PML forms multiprotein complexes referred to as PML nuclear bodies or nuclear domain 10 with several virus gene products: adenovirus type 5, herpes simplex virus type 1, cytomegalovirus (CMV), Epstein-Barr virus, and papillomavirus (13). LCMV infection results in redistribution of PML nuclear bodies to cytoplasmic bodies (1). The PML protein is proapoptotic (13), and translocation of PML bodies to the cytoplasm during arenavirus infection may be involved in the antiapoptotic effect of the virus (3). In transfection experiments, Z associates with PML nuclear bodies, binds directly to PML, and translocates bodies to the cytoplasm (1). We also showed that PML and Z interact with the ribosomal P proteins (P0, P1, P2) in the nucleus of uninfected and infected cells, respectively (2). The P proteins form part of the large ribosomal subunit and are required for protein synthesis (reference 2 and references therein). Their association with Z supports earlier findings of virion-associated ribosomes (12).

Colocalization of ribosomal proteins with PML nuclear bodies and with Z led us to investigate whether Z could affect host cell translation. Several ribosomal proteins and translation factors associate with nuclear structures and have nuclear functions in addition to their cytoplasmic translation functions (26). Eukaryotic translation initiation factor 4E, eIF-4E, is involved in mRNA nuclear cytoplasmic transport, loading selected transcripts onto polysomes and translation initiation (9, 18, 20, 21). In addition to its cytoplasmic distribution, eIF-4E forms nuclear bodies distinct from nucleoli in non-exponentially growing cells (11). eIF-4E transforms cells through preferential transport and translation of selected mRNAs that are normally repressed (20, 21). Overexpression of eIF-4E blocks apoptosis in growth factor-restricted fibroblasts (17), while overexpres-

sion of PML or Z under similar conditions is apoptotic (3). In this report, we determine that Z and eIF-4E associate and counteract each other. Whereas eIF-4E promotes translation of cyclin D1 mRNA (21), Z can repress protein production of cyclin D1 and repression is partially counteracted by additional eIF-4E. Z represses cyclin D1 protein production without affecting protein production of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a transcript which is not affected by eIF-4E. Our view is that Z inhibits cyclin D1 protein production by sequestration of eIF-4E and/or associated translation factors. The mechanism by which these entities counteract each other to regulate specific mRNAs remains to be discovered. We discuss the implications of translational repression for the establishment of chronic infections by LCMV.

MATERIALS AND METHODS

Cell culture. NIH 3T3 (ATCC CRL 1658) were grown and maintained in 10% fetal bovine serum and Dulbecco modified Eagle medium (DMEM; Gibco).

Plasmid constructs. Mammalian overexpression constructs containing Z or ZRINGmut were as described elsewhere (1). eIF-4E was obtained as an expressed sequence tag (ATCC 600222) in pCMVSPORT, which contained the entire coding region of eIF-4E except for the last seven residues.

Recombinant protein production. Z and glutathione *S*-transferase (GST) were produced as reported previously (25). The Z constructs were described by Borden et al. (1). The Z fusion protein was cleaved with thrombin (Boehringer-Mannheim). Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentrations from the optical absorbance value at 280 nm.

Transcription studies with HeLa cell nuclear extract. Transcription studies using HeLa nuclear extract (Promega) were carried out as recommended by the manufacturer. Purified protein or H_2O were added to transcription reactions prior to the incubation step. As a negative control, HeLa cell nuclear extract was heat inactivated for 15 min at 95°C prior to use. RNA production from positive control CMV DNA (Promega) was measured by monitoring incorporation of ^{35}S rUTP (Amersham).

Immunofluorescence and confocal laser microscopy studies. Immunofluorescence methods were as described elsewhere (1, 2). Affinity-purified Z polyclonal antibody (1) and the eIF-4E monoclonal antibody (MAb) (Transduction Laboratories) were used. Appropriate fluorescein isothiocyanate (FITC) conjugate and Texas red secondary antibodies (Jackson Immunoresearch) were used. Fluorescence was observed by using a Leica confocal laser microscope with an excitation at 568 nm (red) or 488 nm (green). The two channels were recorded independently to avoid cross-talk between them. The pinhole was set to 20. Under these conditions, there was no breakthrough of FITC signal into the red channel or vice versa. Experiments were repeated at least twice with at least 500 cells in each sample. Images were overlaid in Photoshop.

Transient transfection. Appropriate constructs were transfected into cells with Lipofectamine or Superfect as directed by the manufacturers (Gibco or Qiagen). After 48 to 72 h, experiments were carried out. The efficiency of transfection was determined by immunofluorescence and confocal microscopy. Our previous studies indicated that there were no significant differences between the constructs used in terms of transfection efficiency and protein production.

Transient transfection and metabolic labeling. After transfection, cells were washed, and methionine-free DMEM (Gibco) was added. Cells were starved for 15 to 30 min. ^{35}S-labeled methionine (Amersham) was added at 20 to 50 μCi/ml, and cells were incubated for 3 h. Cells were resuspended in lysis buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 100 μM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitors as described in the subcellular fractionation section). Total protein concentrations were determined in duplicate by using a Protein Assay kit (Bio-Rad). Then, 20 μg of total protein from each experiment was subjected to SDS-PAGE. Results were observed by autoradiography and analyzed by using NIH Image 1.58 software. In similar experiments, cell lysates were immunoprecipitated after metabolic labeling to monitor the amount of new protein produced as described earlier (21). The same amount of total protein from cell lysates were immunoprecipitated (see below) with cyclin D1, cyclin E (Santa Cruz), or GAPDH (Chemicon) antibodies. Immunoprecipitated samples were subjected to SDS-PAGE, and the results were monitored by autoradiography. Western analysis indicated that equivalent amounts of Z, ZRING, or eIF-4E proteins were produced during transfection and confirmed that the bands studied were cyclins D1, E, or GAPDH as appropriate. In control experiments, vector refers to empty mammalian expression vector (1).

Subcellular fractionation. Cells were fractionated as described earlier (1, 2). After harvesting, cells were washed in cold phosphate-buffered saline, spun, placed in buffer A (110 mM potassium acetate, 2 mM magnesium, 2 mM dithiothreitol [DTT], 10 mM HEPES; pH 7.3) and then spun and resuspended with protease inhibitors and 20 μM cytochalasin B in buffer B (10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 5 mM HEPES; pH 7.3). Typically, protease inhibitors included 2 μg (each) of leupeptin, pepstatin A, and 0.05% aprotinin per ml. Cells were disrupted by passage through 18-, 21-, and 23-gauge needles on ice. Lysates were spun at 1,500 × *g* for 15 min at 4°C to yield a pellet and a supernatant designated the nuclear and cytoplasmic fractions, respectively.

Coimmunoprecipitation studies. Protein-protein interactions were demonstrated by coimmunoprecipitation assays (see reference 6). Cell lysates were mixed with the appropriate antibody: rabbit anti-Z sera (1) or MAb eIF-4E. These antibodies were covalently bound to protein A-Sepharose beads. Z, eIF-4E, or mouse immunoglobulin G (IgG) were immunoprecipitated in separate experiments. Fractions were precleared as described earlier (6). Protein A-antibody beads were added to precleared supernatants and mixed for 2 h at 4°C. Beads were washed three times with IPB buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 100 μM PMSF, and 5 μg of leupeptin, pepstatin A, and 0.05% aprotinin per ml) and three times with modified IPB buffer (0.1% deoxycholate and no Nonidet P-40). Beads were subjected to SDS-PAGE and blotted (Western method) onto Immobolin-P membranes by using enhanced chemiluminescence (Amersham) to visualize the bound antibodies. Blots were probed with eIF-4E antibody or to actin (Sigma). In a previous study (2), antibodies were not covalently attached to protein A-Sepharose beads as described here, and we failed to see the association between eIF-4E and Z. This more sensitive method of immunoprecipitation was employed here.

RNA extraction and analysis. RNA was extracted from transfected NIH 3T3 cells by using Trizol (Life Technologies) according to the manufacturer. Purified RNA was quantitated by spectrophotometry. For Northern analysis, typically 20 μg of RNA was analyzed on agarose-formaldehyde gels. RNA was transferred onto positively charged membrane by using the Northern Max-Plus kit (Ambion). Hybridization was performed by using psoralen-biotin-labeled nonradioactive cDNA probes. Bands were detected by using the Brightstar Biodetect kit (Ambion). Band intensities were measured with NIH Image 1.58 software. Variation in loading was corrected by normalizing values against 28S and 18S rRNA band intensities from the corresponding ethidium-stained gel.

RNA fractionation procedure. The fractionation procedure was as described elsewhere (10a, 21). The RNA distribution was analyzed in three fractions: the cytoplasm (supernatant of the first lysis), the postnuclear fraction (nuclear wash fraction), and the nucleus. Cytoplasmic and postnuclear fractions were considered as cytoplasmic fractions. Initially, RNA levels were measured spectrophotometrically to determine the overall RNA distribution in the cell. The mean and standard deviations (SD) for endogenous total RNA distribution from nine separate transfection experiments were as follows: cytoplasm, 55 ± 14%; postnuclear fraction, 26 ± 9%; and nucleus, 19 ± 10% (with no dependence on the constructs used). These results are similar to those of Rousseau et al. (21), who found 15% of total RNA in the nucleus. Our transfection efficiency was approximately 50% (see transfection section above). For analysis of RNA distribution, we used two reference markers: lysine tRNA and U6 snRNA. The distributions of lysine tRNA were as follows: 65 ± 6%, cytoplasm; 17 ± 2%, postnuclear; and 18 ± 7%, nuclear. Similar to previous reports, 10% of lysine tRNA is in the nucleus (21). The distribution of U6 snRNA was monitored. The distribution in our experiments was 38 ± 11% nuclear, 37 ± 10% cytoplasmic, and 25 ± 5% postnuclear. Rousseau et al. (21) found that 40% of the snRNA was confined to the nucleus. Their report indicates that the large proportion of nonnuclear U6 snRNA is due to its small size (108 nucleotides), allowing it to more readily leak out of the nucleus than larger mRNAs (21). Importantly, the distributions of lysine tRNA and U6 snRNA do not vary with the construct transfected. Thus, the fractionation procedure was not transfection dependent.

Time course of infection and preparation of virions. Stocks of LCMV were used to infect HeLa cell cultures, and virions were purified as described (2). Total cell extracts of uninfected and infected cells were prepared and subjected to Western blot analysis.

RESULTS

eIF-4E colocalizes with Z in infected cells. Many ribosomal components and translation factors have cytoplasmic and nuclear distributions; for example, in resting cells eIF-4E is excluded from the nucleoli (2, 10a, 11). Previously, we have shown that Z and PML associate with the nuclear fraction of the ribosomal P proteins (2), and PML has been shown to interact with eIF-4E (10a). Z's interaction with PML led us to investigate whether Z and eIF-4E could associate in infected cells. In resting cells, eIF-4E has a similar nuclear distribution to PML in addition to its cytoplasmic localization (11). Immunofluorescence in conjunction with confocal laser microscopy indicated that a subset of eIF-4E and Z colocalize. In Fig. 1, NIH 3T3 cells, after 90 h of LCMV infection, were stained with an MAb to eIF-4E, MAb eIF-4E (red; panel B), and an affinity-purified polyclonal antibody to Z (green; panel A), with the image overlay (ov) shown in yellow (panel C). Our pattern of

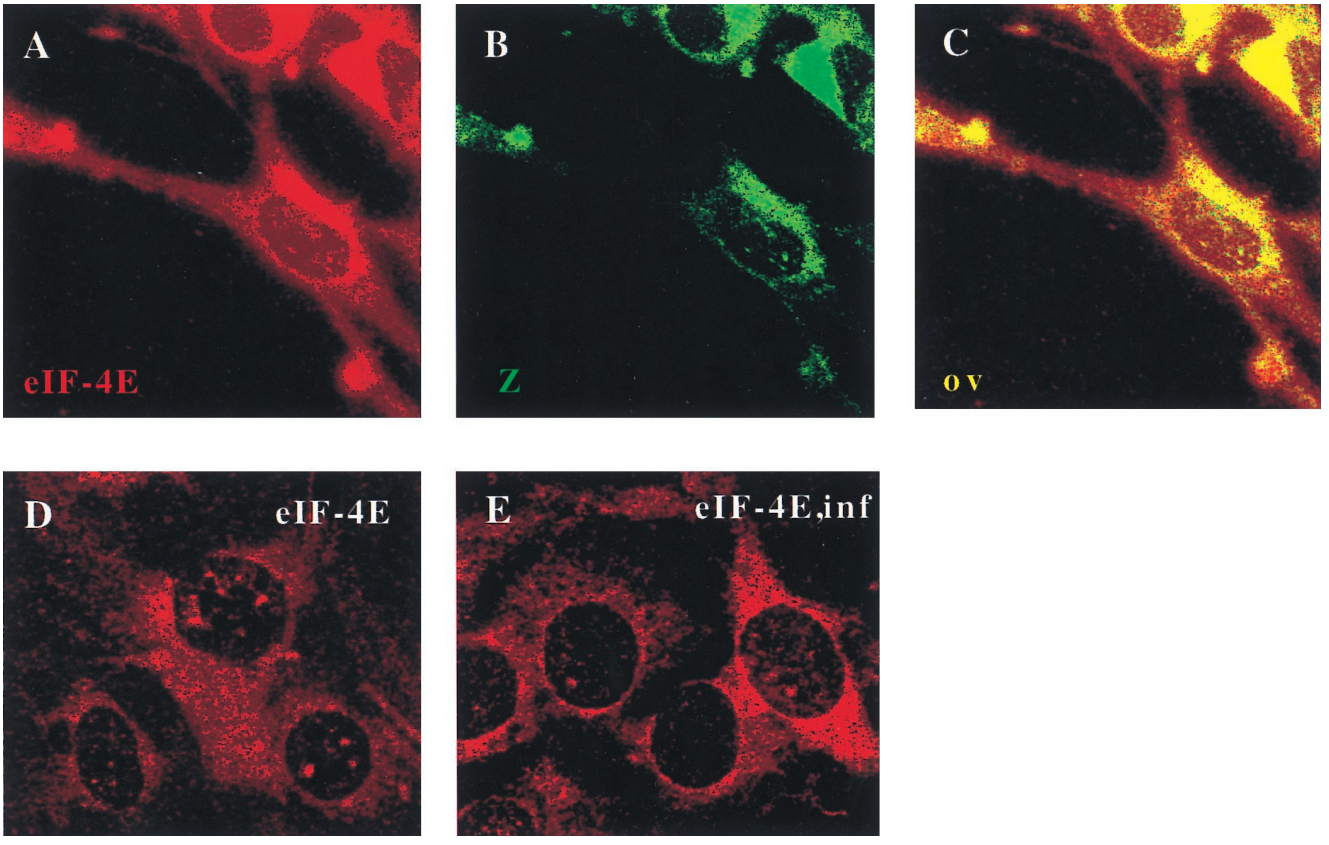

FIG. 1. Z and eIF-4E colocalize. NIH 3T3 cells infected for 90 h with LCMV are shown. Subsequently, cells were stained with affinity-purified Z polyclonal antibody in green (A) and eIF-4E MAb in red (B), and the overlay is shown in yellow (C). In panels D and E uninfected and infected (90 h, inf) cells, respectively, were stained with MAb eIF-4E. The objective is ×100. Panels A to C were further magnified 1.5 times. Panels D and E were magnified 1.8 times. Confocal micrographs represent single slices through the plane of cells. FITC and Texas red channels were recorded independently.

eIF-4E staining is nearly identical to that reported previously, where eIF-4E forms discrete nuclear bodies, excluded from nucleoli, and is distributed throughout the cytoplasm (10a, 11). Immunostaining for Z gave results similar to those reported previously (1, 2), where the majority of Z is cytoplasmic with some nuclear bodies. Z and eIF-4E overlap in discrete nuclear bodies (Fig. 1C). These data indicate that Z and eIF-4E colocalize in a subset of nuclear bodies. The intense diffuse cytoplasmic staining indicates that both Z and eIF-4E overlap but the diffuse pattern makes it difficult to rule out the possibility of coincidental colocalization in the cytoplasm. The cytoplasmic association was confirmed by subcellular fractionation and subsequent immunoprecipitation experiments (see below and Fig. 2).

Previously, we have shown that LCMV infection redistributes the host cell protein PML but leaves other host cell proteins, such as the ribosomal P proteins, unaffected (2). Therefore, we investigated whether the localization of eIF-4E is altered upon LCMV infection. Uninfected cells (Fig. 1D) and cells infected for 90 h (Fig. 1E) were stained only with MAb eIF-4E, and results were observed by confocal microscopy. These data indicate that there is no significant redistribution of eIF-4E upon LCMV infection. In both cases, one observes discrete eIF-4E nuclear bodies which are excluded from the nucleoli as well as an intense diffuse cytoplasmic localization.

Z associates with eIF-4E. To determine whether Z and eIF-4E associate physically, we carried out immunoprecipitation studies in transfected cells. These studies also allowed us to determine whether the colocalization observed between Z and eIF-4E was real or coincidental in the cytoplasm. NIH 3T3 cells were transfected with Z or ZRINGmut constructs. In ZRINGmut, two of the cysteines in the RING were mutated to Phe and Gly, resulting in an unfolded RING (1, 4). The Z protein was immunoprecipitated from nuclear and cytoplasmic fractions with the Z antibody and Western blots probed with MAb eIF-4E. Figure 2 shows that both Z and ZRINGmut associate with eIF-4E. Both mutant and wild-type Z association with eIF-4E occurs in nuclear and cytoplasmic fractions, a finding consistent with our confocal results (Fig. 1). The immunoprecipitation and immunofluorescence data indicate that Z interacts with eIF-4E, but we cannot distinguish whether this is a direct or indirect interaction. As a control for specificity, blots were probed with an antibody to actin, which did not associate with Z or ZRINGmut (Fig. 2A and B). Similarly, cells immunoprecipitated with mouse IgG showed no eIF-4E precipitated (Fig. 2C). The specificity of the commercially obtained MAb eIF-4E was verified. Samples of total cell lysates from untransfected NIH 3T3 cells or from cells transfected with the eIF-4E gene were prepared where gel samples were normalized to the same number of cells. Samples were subjected to SDS-PAGE, blotted, and probed with MAb eIF-4E (Fig. 2D). eIF-4E is produced normally in NIH 3T3 cells (3T3 lane), and a single band is observed at the expected molecular mass (25 kDa). In cells overexpressing eIF-4E (3T3/eIF-4E lane), the signal is significantly greater than in the untransfected cells. These data confirm the specificity of MAb eIF-4E.

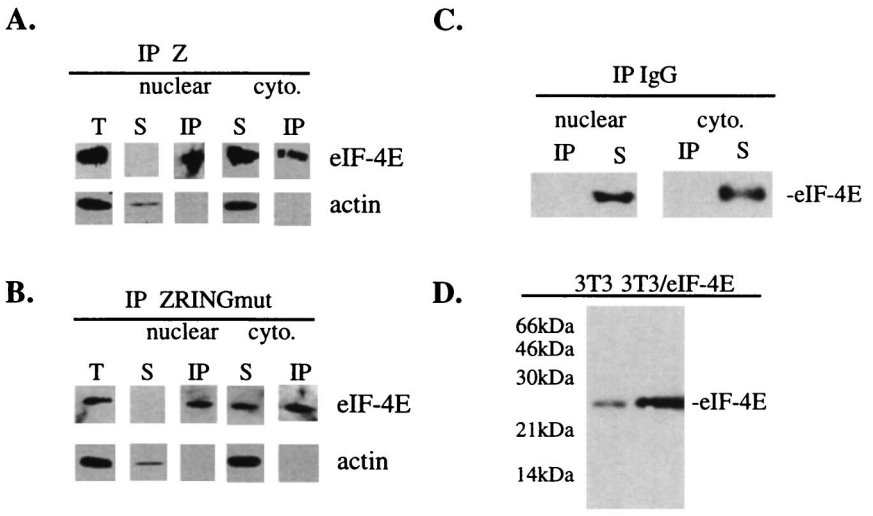

FIG. 2. Z and eIF-4E associate physically. (A and B) Z and ZRINGmut coimmunoprecipitate eIF-4E. NIH 3T3 cells were transfected with Z (A) or ZRINGmut (B). The resulting lysates were immunoprecipitated with Z antisera. Nuclear, nuclear fraction; cyto., cytoplasmic fraction; total, total cell lysate; S, supernatant after immunoprecipitation (IP). Western blots were probed as indicated. (C) Cell lysates were immunoprecipitated with mouse IgG as a negative control. Western blots were probed with eIF-4E or actin as indicated. N, nuclear fraction; cyto., cytoplasmic fraction; T, total; S, supernatant after immunoprecipitation. (D) The specificity of the commercially obtained eIF-4E antibody (MAb eIF-4E) was assessed. Western blots of control cells lysates (3T3) or cells transfected with eIF-4E (3T3/eIF-4E) were probed with MAb eIF-4E.

Consistent with our immunofluorescence results, Z and eIF-4E associate in both the nucleus and cytoplasm. These data are consistent with our previous findings that Z associates with ribosomal components. However, eIF-4E is usually found in complexes with other eIFs (18). Therefore, although we have established that these proteins interact, we have not established that they interact directly. It is possible that Z directly associates with another member of the eIF complex. Nonetheless, our immunoprecipitation and immunofluorescence results clearly indicate that Z associates with eIF-4E in the cytoplasm and in discrete bodies in the nucleus. The association of Z with eIF-4E and the ribosomal P proteins has important implications to the function of this protein (see below).

Z decreases protein production selectively in cell culture. These data and previous work indicate that Z associates with proteins involved in protein synthesis, including P0, P1, and P2 (2) and eIF-4E (the present study). These associations raise the distinct possibility that Z exerts an influence on protein synthesis. In support of this possibility, the majority of Z is cytoplasmic in transfected and infected cells (1). Therefore, we assessed the effect of Z overexpression on protein production in cell culture. NIH 3T3 cells were transiently transfected with Z, ZRINGmut, or vector. [^{35}S]methionine (^{35}S-Met) incorporation into total protein was monitored by autoradiography. Cells were labeled for 3 h prior to harvesting. Western analysis followed by measurement of band intensities indicated that equal levels of Z and ZRINGmut proteins were produced in the relevant transfection experiments. The average signal intensities from three separate autoradiographs are shown (Fig. 3A). The transfection efficiency was approximately 50% (see Materials and Methods). Slightly more ^{35}S-Met was incorporated in cells transfected with the ZRINGmut construct than Z, with differences in incorporation of less than 10%. Notably, levels of ^{35}S-Met incorporation were not significantly different in cells transfected with vector or Z. Thus, Z does not significantly reduce total protein production in cell culture.

In cell culture, eIF-4E is known to affect the production of selected proteins. Thus, we investigated whether Z acted similarly. Levels of eIF-4E protein are known to increase protein production of cyclin D1 but not GAPDH by preferential nuclear cytoplasmic transport of cyclin D1 mRNA and preferential loading of transcripts onto polysomes (20, 21). eIF-4E does not alter overall levels of either mRNA (20, 21). This differential action of eIF-4E is thought to result from the structures of untranslated regions (UTRs) of GAPDH and cyclin D1 mRNAs. We monitored the production of cyclin D1 and GAPDH in cells expressing Z, ZRINGmut, or eIF-4E. We monitored the production of another cyclin, E, to determine if any effects were cyclin D1 specific. Cells were transfected and labeled with ^{35}S-Met as before. Total cell lysates were normalized for total protein concentration, and the same amount of protein was used for each immunoprecipitation experiment. In these experiments, the total levels of each protein were not monitored, but the levels of protein produced during the ^{35}S-Met labeling step were measured. Thus, reduced ^{35}S-Met-labeled protein levels indicate lower levels of protein production during labeling. The immunoprecipitated protein was analyzed by SDS-PAGE and autoradiography. Band intensities for cyclins D1 and E were normalized to the intensity of the respective GAPDH bands from the same cells. Autoradiographs are shown in Fig. 3B, where values for intensities are given below the corresponding band. Western analysis confirmed that the bands corresponded to the appropriate protein. Analysis confirmed that similar amounts of wild-type and mutant Z and eIF-4E proteins were produced in the relevant transfections.

These experiments indicate that Z suppresses production of cyclin D1 and E relative to vector transfected cells (Fig. 3B). Further, production of cyclins D1 and E is lower in cells expressing Z than in cells expressing ZRINGmut (Fig. 3B). Cyclin D1 is reduced nearly fivefold, and cyclin E is reduced eightfold by Z versus ZRINGmut. Importantly, Z inhibits cyclin D1 and E production but not GAPDH production in the same cells. Thus, Z has a similar pattern of action to eIF-4E but acts in a contrary manner, repressing production of proteins which eIF-4E alone would upregulate.

If the effect of Z were dependent on eIF-4E, one would expect that eIF-4E overexpression could counteract Z's repression. Consistent with previous reports (21), eIF-4E increases

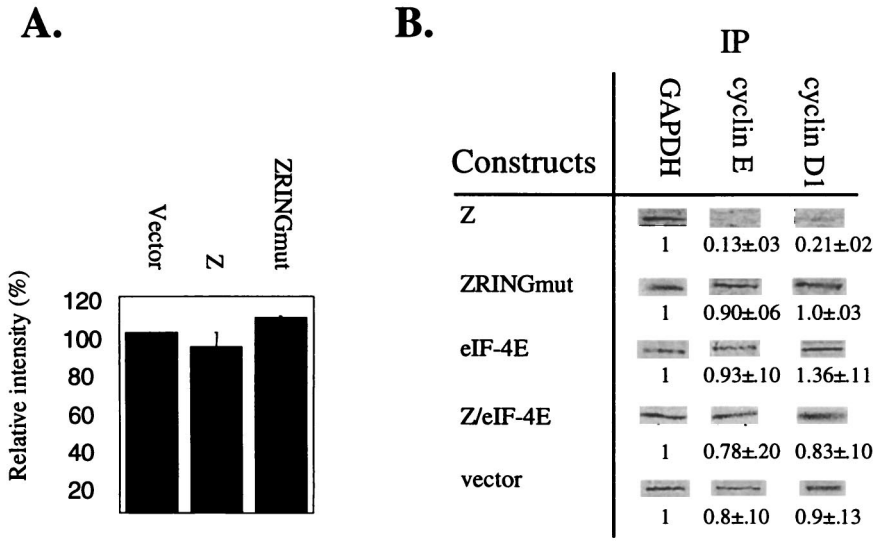

FIG. 3. (A) ^{35}S-Met incorporation in cells overexpressing the indicated constructs. Total protein production was measured by autoradiography, and band intensities were quantitated. Mean band intensities and SDs for each transfection taken from three independent experiments are shown. (B) Z affects cyclin D1 and E production. Cells were transfected and metabolically labeled. The same amount of total protein from cell lysates was immunoprecipitated (IP) with antibodies to cyclins D1, E, or GAPDH as indicated. Immunoprecipitated protein was monitored by autoradiography. Values were normalized to those of GAPDH in each experiment. Mean band intensities and standard errors are given below each band. Results for three experiments were quantitated.

production of cyclin D1 relative to vector or Z and similarly for cyclin E. In cells overexpressing both Z and eIF-4E, eIF-4E overexpression alleviates repression of cyclin D1 and E production by Z. eIF-4E coexpression with Z increases production of cyclin D1 protein by fourfold relative to Z expression alone and similarly for cyclin E by eightfold. However, expression of cyclin D1 levels do not recover to levels observed with eIF-4E expression alone. These observations suggest that in cell culture selective repression of protein production by Z is mediated at least in part through eIF-4E. Importantly for all experiments, the levels of GAPDH protein obtained were nearly identical. eIF-4E protein levels are not reduced by Z overexpression. No protein degradation was observed in these studies.

Z does not alter transcription. It is essential to establish if these effects of Z and eIF-4E are transcriptional or posttranscriptional in cell culture. Northern analysis was carried out to ascertain whether RNA levels were reduced in cells expressing Z. RNA was extracted from cells transfected with Z, ZRINGmut, or vector, and production of GAPDH and cyclin D1 mRNA was monitored by Northern analysis. mRNA levels were quantified by measurement of band intensities and then normalized to the value of band intensities for the 28 and 18S rRNA in the corresponding ethidium-stained gels. Normalized cyclin D1 values are shown in Fig. 4. Similar results were observed for GAPDH mRNA. The small variations in cyclin D1 mRNA production are not sufficient to explain the five- to eightfold reduction we observed at the protein level. Further, in contrast to the suppression of cyclin D1 protein production in Z-transfected cells, RNA production of cyclin D1 was slightly higher in cells transfected with Z than control cells. Thus, the effect of Z on cyclin D1 protein production is posttranscriptional.

To confirm the above Northern results and to demonstrate that Z does not have a general effect on the transcriptional machinery, we monitored the effect of Z on RNA production in HeLa nuclear extract (Fig. 4B). Transcription from the CMV immediate-early promoter was assessed by monitoring the incorporation of [^{35}S]rUTP by autoradiography. Addition of purified Z protein does not significantly decrease the levels of RNA produced compared with the effect of buffer, GST, or bovine serum albumin (Fig. 4B). For negative controls, extracts were heat inactivated prior to the addition of template (Fig. 4B, lane 4), resulting in no production of RNA. As expected, no signal was observed when no extract was added or if unlabeled rUTP was used (Fig. 4B, lanes 6 and 7). Thus, repression of protein production is not due to transcriptional inhibition because RNA is produced in the presence of Z. Furthermore, the RNA production indicates that there is no significant RNase activity in our protein preparations. Our data suggest that, in HeLa nuclear extract systems, Z does not reduce production and stability of the transcripts studied.

eIF-4E modulates nuclear cytoplasmic mRNA transport of cyclin D1 (21). We have shown that Z binds the PML protein (1). Further, PML, a primarily nuclear protein, represses production of cyclin D1 protein by nuclear retention of cyclin D1 mRNA through an eIF-4E-mediated mechanism (10a). Thus, we investigated whether Z affects cyclin D1 mRNA distribution as a possible mechanism for Z-induced suppression of cyclin D1 protein production (Fig. 4C). Cells transfected with the appropriate DNA were fractionated as described (21), resulting in the preparation of nuclei free of cytoplasmic contamination. Several experiments were carried out to assess the quality of the fractionation (see Materials and Methods).

We examined the effect of overexpressing Z and ZRINGmut on the subcellular distribution of endogenous cyclin D1 and GAPDH mRNAs in the same cells. RNA from each fraction was analyzed by Northern blotting. Values were normalized to 28S and 18S rRNA band intensities to correct for gel loading errors and corrected for subcellular distribution (Fig. 4C) according to the method of Rousseau et al. (21). There is no significant difference in the levels of nuclear GAPDH or cyclin D1 mRNA regardless of the protein(s) overexpressed (Fig. 4C). Thus, unlike PML, Z does not reduce cyclin D1 protein production by retention of cyclin D1 mRNA in the nucleus. This finding is consistent with the majority of Z protein being

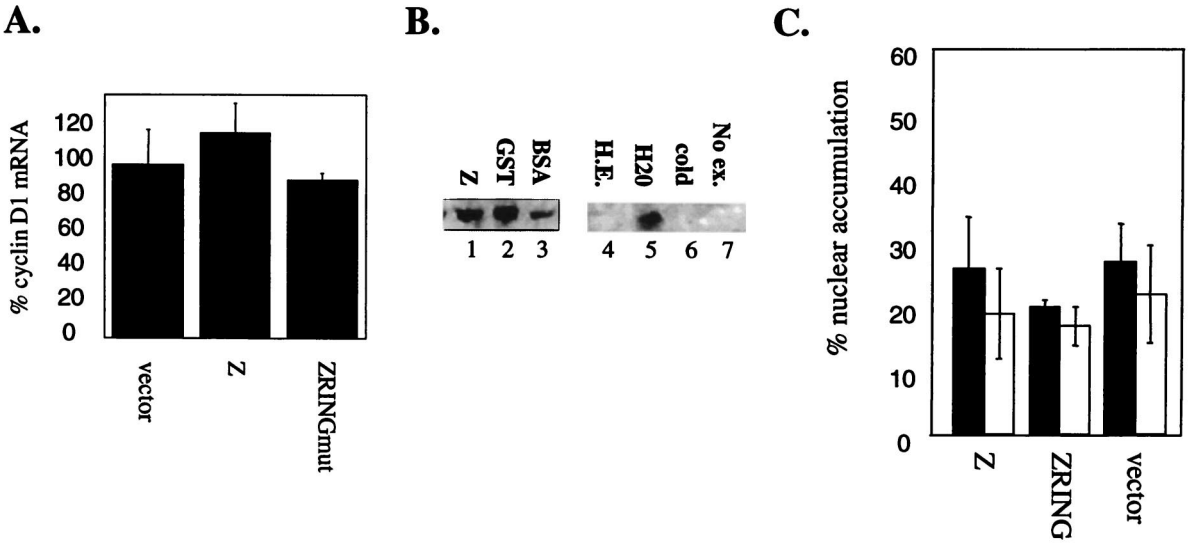

FIG. 4. Z does not inhibit transcription. (A) Z overexpression does not reduce levels of cyclin D1 transcripts. Total cyclin D1 mRNA levels were determined for cells transfected with the indicated constructs by using Northern analysis. Cyclin D1 mRNA levels were normalized to 28S and 18S rRNA band intensities. Experiments were carried out in duplicate and varied as shown. (B) Z does not alter transcription. CMV immediate-early promoter fragment (Promega) was transcribed in the presence of 30 μM (each) Z, GST, bovine serum albumin, or H_2O. RNA production was monitored by incorporation of [^{35}S]rUTP and autoradiography. The second panel shows the effect of heating extract prior to the assay (H.E.). "Cold" refers to the use of unlabeled rUTP, and "No ex." means no extract was used. (C) Z does not alter the distribution of cyclin D1 mRNA. RNA distribution in cellular fractions overexpressing the listed constructs was monitored. The percentage of nuclear cyclin D1 (solid bars) or GAPDH (open bars) RNA is given. Values of band intensities in each lane were normalized to band intensities of 28S and 18S rRNA to correct for differences in loading. RNA levels were normalized to the distribution of total RNA in the cell. Experiments were carried in duplicate and varied by the percentage given.

found in the cytoplasm (1). These results suggest that Z affects cyclin D1 production at the translational level.

LCMV downregulates production of cyclins D1 and E but not eIF-4E. Since cyclin D1 and E protein levels are reduced in cells expressing Z, we expect to see a reduction in these proteins during LCMV infection. Protein levels were monitored by Western analysis. eIF-4E and GAPDH protein levels were also monitored (Fig. 5). Blots of extracts from infected or uninfected HeLa cells were probed with the appropriate antibody. Levels of GAPDH and eIF-4E changed little during the time course. eIF-4E is present in the virion lane (V), indicating that it is incorporated into virions, whereas GAPDH was not. Cyclin E is downregulated starting at 48 h postinfection (p.i.). Cyclin D1 is downregulated in the first 24 h p.i. but increases dramatically at 96 h p.i. This increase in cyclin D1 at 96 h was observed in three independent experiments. Notably, the same lysate preparations were used for monitoring production of

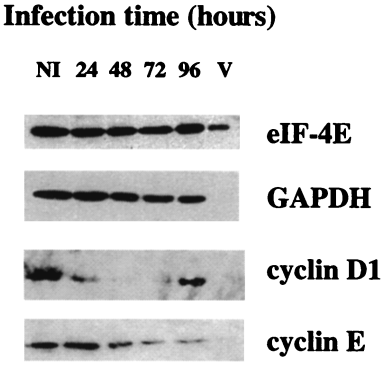

FIG. 5. Western blot analysis of infected HeLa cells. Times indicate hours p.i., and "V" refers to virions. Blots were probed as indicated.

eIF-4E, GAPDH, and both cyclins. Therefore, the differences seen for cyclin D1 versus cyclin E are not dependent on lysate preparation. Neither cyclin is incorporated into virions. There was no evidence of protein degradation on these blots.

DISCUSSION

Little is known about the host cell molecules required for arenavirus infection. While arenaviruses replicate in the cytoplasm, the nucleus is required for replication. Others have reported that cells enucleated prior to 12 h after infection cannot complete virus replication (reviewed in reference 23). Additionally, arenaviruses are thought to contain ribosomes due to their appearance in electron micrographs and the ability of virus to polymerize radioactive amino acids (12). In support of this observation, we have shown that the ribosomal P proteins (2) and eIF-4E (the present study) are incorporated into virions and that Z can physically associate with both the P proteins (2) and eIF-4E. The association of Z with host cell translational machinery led us to investigate whether it affected protein synthesis. We show that the Z protein can repress specific protein production at the posttranscriptional and post-RNA transport levels in cell culture. Expression of eIF-4E in cell culture partially alleviates repression by Z, indicating that decreased protein production is in part mediated by eIF-4E. Repression may be mediated by sequestration of additional ribosomal components such as the P proteins and other components of the eIF-4E complex.

Our studies indicated that mutation of the first zinc-binding site of the RING domain does not alter Z's ability to coprecipitate eIF-4E but does disrupt its translational suppression action. Thus, eIF-4E association alone is not sufficient for Z's repression activity. This leaves two questions: (i) how are both Z and ZRINGmut associating with eIF-4E and (ii) why has ZRINGmut lost the ability to repress translation? First, other

studies of RING domains indicate that proteins which require one RING zinc-binding site can bind partner proteins regardless of the integrity of the other site (14, 19). Thus, Z may be able to bind eIF-4E through either the second zinc-binding site of its RING domain, its proline-rich region, or its N-terminal domain. Second, the ability of Z to repress translation requires an intact first zinc-binding site. We show that translational repression is eIF-4E dependent because additional eIF-4E alleviates repression. It appears that the first zinc-binding site must make crucial protein contacts with other partners, e.g., P proteins, which are also required for translation. For instance, eIF-4E is known to exist in complexes with other eIF proteins; thus, once Z associates with eIF-4E it probably contacts other components of the eIF complex. Our results suggest that the first zinc-binding site must make contacts with other proteins which are vital for Z's ability to repress translation. Additionally, recent studies have implicated RINGs in ubiquitination complexes (5). However, Z was not active in these assays (K. L. B. Borden, A. Chen, and Z. Q. Pan, manuscript in preparation), and therefore reduced protein production is unlikely a result of ubiquitination followed by degradation.

In cell culture, Z affects production of proteins in an eIF-4E-dependent manner. Overexpression of Z results in decreased protein production of cyclin D1, an eIF-4E-sensitive transcript. In contrast, Z does not affect production of GAPDH, an eIF-4E-insensitive transcript. The nature of the untranslated regions of these transcripts is thought to be the basis for eIF-4E and therefore Z sensitivity (21). The effects of Z are posttranscriptional since we do not see reduced RNA production in any of our assays. Coexpression of Z and eIF-4E results in recovery of cyclin D1 and E protein production. The importance of this interaction during infection is highlighted by our results showing that production of cyclins D1 and E is reduced during the time course of infection and by the fact that eIF-4E is incorporated into virions.

The ability of eIF-4E to modulate protein production selectively in cell culture relies on its presence in the nucleus in order to influence transport of selected transcripts to the cytoplasm and, in the cytoplasm, to preferentially load certain transcripts onto polysomes (21). Subcellular fractionation in conjunction with Northern analysis indicates that Z does not interfere with the transport function of eIF-4E. In contrast to PML, which sequesters cyclin D1 transcripts in the nucleus in uninfected cells (10a), Z does not. In cell culture, Z forms nuclear and cytoplasmic bodies with PML (1). It has been demonstrated that PML bodies associate with transcripts (13). It is possible that when Z associates with PML and translocates PML bodies to the cytoplasm, some of the transcripts in the PML and Z bodies remain sequestered and thus unavailable for association with the translational machinery. Perhaps Z also interferes with the ability of eIF-4E to associate with and preferentially load transcripts onto polysomes (21). Therefore, it seems likely that protein production in cell culture would be repressed by Z's association and potential disruption of PML and eIF-4E bodies.

Arenaviruses such as LCMV and Lassa fever virus are noncytopathic in cell culture and easily establish chronic infections in their murine hosts (23). The NP protein can reduce Z's translational repression (unpublished observations), indicating that interplay between viral proteins may modulate Z function and perhaps be necessary for establishing chronic infection. Chemical cross-linking studies show that Z and NP are closely associated in the virion (22). Previously, we showed that overexpression of Z reduces cell survival in serum-starved fibroblasts (3). In contrast, LCMV-infected fibroblasts survive serum withdrawal better than the uninfected controls (3). In our model, overexpression of Z alone would result in marked decrease in cyclins D1 and E and other eIF-4E-sensitive transcripts. Cyclins D1 and E are essential for the G_1/S transition of the cell cycle (10), and Z protein production could halt cell cycle progression by arresting cells in G_1. Thus, Z expression could account for the slower growth in infected cells. However, downregulation of cyclins may reduce cell viability. During LCMV infection, other viral proteins, such as the NP protein, would be present in excess of Z and could modulate the action of Z, thereby preventing the demise of the host cell.

Acquisition of translation machinery such as eIF-4E and the P proteins (2) by the virus could explain how viral mRNA is selectively translated over cellular mRNA. Arenavirus mRNAs contain highly structured UTRs (23) and thus may require eIF-4E for efficient translation. Sequestration of eIF-4E by Z would block the translation of cellular- and viral-UTR-containing mRNA. In fact, increasing Z expression diminishes expression of viral envelope protein (23). A complete virion can be produced within 6 to 10 h after the virus enters the cell, with the production of viral RNA limiting the assembly of particles (23). We speculate that only later in the viral life cycle, when available NP wanes and levels of Z protein are high, is Z sufficiently abundant to interact with eIF-4E and shut off translation of UTR-containing mRNA. Such a self-regulating mechanism could account for the noncytopathic nature of arenaviruses in that they limit their own translation so as not to drastically impact the health of the host cell.

In summary, we demonstrate that the arenavirus Z protein inhibits protein production through its interactions with host cell translational machinery. This action of Z is dependent on the integrity of its RING domain. We show that in cell culture Z represses production of certain proteins without altering RNA production and that selectivity of repression appears to be mediated through its interaction with eIF-4E. The resulting decrease in cyclins D1 and E seen in cells overexpressing Z, as well as in infected cells, suggests a mechanism for slower growth seen in infected cells and perhaps elucidates a viral strategy for establishing chronic infection.

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