Efficient Induction of Nuclear Aggresomes by Specific Single Missense Mutations in the DNA-binding Domain of a Viral AP-1 Homolog*§

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Nuclear aggresomes induced by proteins containing an expanded polyglutamine (polyQ) tract are pathologic hallmarks of certain neurodegenerative diseases. Some GFP fusion proteins lacking a polyQ tract may also induce nuclear aggresomes in cultured cells. Here we identify single missense mutations within the basic DNA recognition region of Bam HI ZEB virus replication activator (ZEBRA), an Epstein-Barr virus (EBV)-encoded basic zipper protein without a polyQ tract, that efficiently induced the formation of nuclear aggresomes. Wild-type (WT) ZEBRA was diffusely distributed within the nucleus. Four non-DNA-binding mutants, Z(R179E), Z(R183E), Z(R190E), and Z(K178D) localized to the periphery of large intranuclear spheres, to discrete nuclear aggregates, and to the cytoplasm. Other non-DNA-binding mutants, Z(N182K), Z(N182E), and Z(S186E), did not exhibit this phenotype. The interior of the spheres contained promyelocytic leukemia and HSP70 proteins. ZEBRA mutants directly induced the nuclear aggresome pathway in cells with and without EBV. Specific cellular proteins (SC35 and HDAC6) and viral proteins (WT ZEBRA, Rta, and BMLF1) but not other cellular or viral proteins were recruited to nuclear aggresomes. Co-transfection of WT ZEBRA with aggresome-inducing mutants Z(R183E) and Z(R179E) inhibited late lytic viral protein expression and lytic viral DNA amplification. This is the first reported instance in which nuclear aggresomes are induced by single missense mutations in a viral or cellular protein. We discuss conformational changes in the mutant viral AP-1 proteins that may lead to formation of nuclear aggresomes.

Sequestration of aggregates of misfolded proteins within prominent intracellular inclusion bodies (IBs)² is a pathological hallmark of several neurodegenerative diseases and prion dis-

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2 The abbreviations used are: IB, inclusion body; EBV, Epstein-Barr virus; bZIP, basic zipper; PML, promyelocytic leukemia; aa, amino acid(s); Z, ZEBRA; HCMV, human cytomegalovirus; CREB, cAMP-response element-binding protein; ZEBRA, Bam HI Z E B virus replication activator; PLIC, protein linking integrin-associated protein to cytoskeleton; BZKO, Bam HI Z knock-out; gZ, genomic ZEBRA gene; Rta, R transactivator.

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Although clinically and pathologically distinct, a single unifying feature of polyQ diseases is the formation of aggresome-like IBs located primarily in the nucleus. These intranuclear aggresomes contain aggregates of misfolded mutant proteins and are invariably linked to end stage neurodegenerative disease.

The mechanisms involved in the formation of intranuclear aggresomes are not understood. Studies in transgenic mice and in cell culture have shown that nuclear IBs are enriched in chaperones and proteasomal subunits (12, 13). Differences exist between the nuclear and cytoplasmic aggresomes. Unlike the solitary inclusion body at the microtubule-organizing center characteristic of cytoplasmic aggresomes, intranuclear aggresomes exist as multiple discrete inclusions. The absence from the nucleus of an extensive network of microtubules, which is essential to cytoplasmic aggresome formation, would indicate differences in the mechanisms of nuclear aggresome formation. The formation of nuclear aggresomes by a variety of different mutant proteins is invariably accompanied by the redistribution and recruitment of promyelocytic leukemia (PML) protein. Because PML resides exclusively within the nucleus, it is unlikely that it would be recruited to cytoplasmic aggresomes at the microtubule-organizing center via microtubules.

The link between polyQ expansions and the formation of intranuclear aggresomes initially suggested that their formation was induced by mechanisms that specifically respond to polyQ tract expansions. However, several mutant proteins that lack polyQ domains activate the formation of nuclear aggresomes that share characteristics of nuclear aggresomes induced by mutant polyQ proteins. GFP-170*, consisting of GFP fused to a portion of the Golgi complex protein 170, induced the formation of nuclear aggresomes that recruited PML protein, the chaperones HSP70 and HDJ2, and proteasomal subunit-α (14–17). Another mutant protein, REDΔ87–113GFP, which lacks a polyQ domain and contains a polar zipper repeat domain, was also shown to induce nuclear aggresomes that resembled those formed by mutant polyQ-containing ataxin-1 (18). Cellular and environmental stresses, such as generation of reactive oxygen species and irradiation, have also been shown to induce the formation of nuclear aggresomes that resemble those generated by mutant polyQ proteins (19, 20).

The tight association between polyQ expansions and nuclear aggresomes indicates a degree of specificity in the mechanisms that activate the nuclear aggresome pathway. However, the induction of this pathway by at least two non-polyQ mutant GFP fusion proteins and by environmental stresses also indicates that the sensing and activation mechanism is not restricted to expansions of polyQ tracts. The mutations introduced into the two previously described non-polyQ protein inducers of the nuclear aggresome pathway each consist of a large deletion of the native protein and the fusion of a 238-amino acid GFP moiety. These chimeric mutants thus do not elucidate the precise nature of the alterations recognized by the sensing mechanism. It would be desirable therefore to limit the mutation recognized by the nuclear aggresome pathway to as small a region as possible, ideally a single amino acid change. This would enhance the opportunity to specifically characterize the nature of the conformational change that activates the nuclear aggresome pathway. In this study, we describe the induction of nuclear aggresomes by the introduction of single missense mutation in a non-polyQ protein, Bam HI Z.E.B virus replication activator (ZEBRA). Furthermore, by analyzing several missense mutations at various positions in ZEBRA, we demonstrate the utility of this system in investigating the precise changes responsible for activating the nuclear aggresome pathway.

ZEBRA, encoded by the Epstein-Barr virus (EBV) BZLF1 gene, is the major regulator of the lytic phase of EBV. ZEBRA is a 245-amino acid (aa) protein that belongs to the basic zipper (bZIP) family of transcription factors that includes the AP-1 transcription factors c-Fos and c-Jun. ZEBRA can be modified by phosphorylation (21) and SUMOylation (22). ZEBRA homodimerizes and binds DNA at canonical AP-1 sites (TGAGTCA) via the bZIP domain (23, 24). However, unlike c-FOS, ZEBRA also binds degenerate AP-1-like heptad sequences with the consensus sequence 5′-T(T/G)/(T/A)G(T/C)(G/C/A)-3′ (25), termed “ZEBRA-responsive elements.”

The bZIP domain of ZEBRA encompasses the basic DNA-binding domain (aa 178–194) and the dimerization domain (aa 195–225) (26). Nine of 17 aa in the DNA-binding domain are arginines or lysines. The crystal structure of the S186A/C189A mutant of ZEBRA bound to an AP-1 site showed a continuous α helix between aa 175 and 221 (27). Other bZIP proteins whose structures have been solved also showed a long continuous α helix encompassing their bZIP domains (28, 29).

Heston et al. (30) analyzed an extensive series of single amino acid substitutions introduced throughout the basic domain of ZEBRA. Of these mutants, 27 retained the capacity to bind DNA yet showed specific defects in the ability to activate different phases of the lytic cycle. These defects in transcriptional activation were accompanied by distinct changes in the localization of ZEBRA and in the capacity to induce the formation of replication compartments (31). Nineteen of the 46 mutations abolished the ability of ZEBRA to bind ZIIIB, a high affinity ZEBRA-responsive element, as seen by mobility shift assay. Each of these 19 DNA binding-defective mutants was also incapable of activating the lytic cycle of EBV. The initial impetus of the current study was to observe the localization of several mutants of ZEBRA that were severely impaired in DNA binding.

We report here that specific single missense mutations within the basic domain of ZEBRA induce the formation of nuclear aggresomes, which strongly sequester the mutant protein. The aggresomes contain two identifying components of nuclear aggresomes, HSP70 and PML protein. These aggresomes have the characteristic ability to recruit and sequester other specific non-mutated cellular proteins, including HDAC6 and SC35, and viral proteins, including those encoded by BZLF1, BRLF1, and BMLF1 genes. Transfection of aggresome-inducing mutants of ZEBRA strongly inhibits the lytic cycle and replication of EBV. A review of those missense mutations that can induce the nuclear aggresome response suggests that the mutations responsible for inducing aggresomes may be limited to conversion of a basic amino acid (arginine or

\[ \text{ARG} \rightarrow \text{GLY} \]
lysine) to an acidic amino acid as well as additional contributing factors, such as the absence of contact of ZEBRA with the phosphate backbone of DNA.

**EXPERIMENTAL PROCEDURES**

*Construction of Mutations in EBV bzlf1 Gene—* The procedure for construction of the bzlf1 mutants has been described (30). The 10 mutants analyzed in this study were as follows: K178D (AAG to GAC), K178E (AAG to GAA), R179E (CGG to GAG), N182K (AAT to GAG), N182E (AAT to GAG), R183E (CGG to GAG), S186E (TCC to GAG), R190E (CGG to GAG), K194D (AAG to GAG), and K194E (AAG to GAA).

**Cell Lines—** 293 cells are a human embryonic kidney cell line immortalized by the early region of adenovirus (32). 2089 cells are a 293 cell line stably transfected with a bacmid containing the full-length EBV genome and a hygromycin B resistance gene (33). Bam HI Z knockout cells are a 293 cell line stably transfected with an EBV bacmid in which the BZLF1 gene has been inactivated by insertion of a hygromycin resistance cassette (34). 293 cells were maintained in RPMI 1640 complete medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin-streptomycin, and 1 µg/ml amphotericin B. 2089 and BZKO cells were maintained in RPMI 1640 complete medium containing 100 µg/ml hygromycin B (Calbiochem).

**Antibodies—** In immunofluorescence experiments, ZEBRA was detected with a rabbit polyclonal antibody (S1605) or a mouse monoclonal antibody (BZ1) (35). S1605 was prepared from rabbits immunized with full-length ZEBRA protein that was expressed in *Escherichia coli* from a PET22b vector containing the BZLF1 cDNA and purified over a nickel-agarose column. Early antigen-diffuse was detected using the mouse monoclonal antibody R3.1 (36). SC35, PML, HSP70, and nucleolin proteins were detected using commercially available murine monoclonal antibodies (Abcam ab11826, Santa Cruz Biotechnology sc-966, Abcam ab2787, and Abcam ab13541). HDAC6 was detected using a commercially available rabbit polyclonal antibody (Santa Cruz Biotechnology sc-11420). In immunoblot experiments, ZEBRA was detected using a rabbit polyclonal antibody described previously (21). Rta and BRLF2 protein were detected using rabbit polyclonal antisera described previously (30). BBRF3 was detected with a rabbit polyclonal antibody (S1931-1) raised against full-length BBRF3 protein expressed and purified from *E. coli*. Lamin B was detected using a commercially available goat polyclonal antibody (Santa Cruz Biotechnology sc-6216). Secondary antibodies used in immunofluorescence experiments were purchased from Jackson ImmunoResearch Laboratories: FITC-sheep anti-mouse IgG (515-095-062), Texas Red-donkey anti-rabbit IgG (711-075-152), FITC-donkey anti-goat IgG (705-095-147), Rhodamine Red X-donkey anti-rabbit IgG (711-295-152), and DyLight 549-donkey anti-rabbit IgG (711-055-152).

**Indirect Immunofluorescence—** 2089 and 293 cells grown on glass coverslips were transfected with plasmid DNA using dimethyl sulfoxide Rosenthal inhibitor ether-C reagent (Invitrogen). After 8 h, the transfection reagent was replaced with growth medium. Forty-two hours after transfection, a time previously determined to be adequate for detection of lytic viral DNA replication, cells were fixed in methanol for 30 min at −20°C, washed with PBS, and incubated in blocking solution (10% human serum in PBS) for 1 h at room temperature. Cells were stained with primary antibody diluted in blocking solution for 1 h at room temperature in humidified chambers. Cells were washed with PBS and then incubated with secondary antibody diluted 1:200 in blocking solution for 1 h at room temperature in opaque humidified chambers. Cells were washed with PBS, briefly rinsed in distilled H2O to remove salts, and then mounted on glass slides using Vectashield mounting medium (Vector Laboratories). A Zeiss LSM510 confocal laser scanning microscope was used to obtain digital images of fluorescence and transmitted light. Excited fluorophore emissions were collected either individually or simultaneously under detection settings that reduced cross-talk between the green and red channels to non-detectable levels.

**Transfection and Immunoblotting for Detection of EBV Lytic Cycle Proteins—** BZKO cells were transfected by DMRIE-C reagent (Invitrogen). Cells initially plated at 2 × 10^5 cells/well of a 6-well plate were transfected after 4–5 days when they were 80% confluent. Constant amounts of wild-type (WT) genomic EBV lytic cycle proteins were detected using increased amounts of Z(R183E) or Z(R179E) plasmids in 3 ml of Opti-MEM containing 36 µl of DMRIE-C reagent. After 72 h of incubation at 37°C, BZKO cells were removed from the plastic surface by forceful pipetting, pooled, centrifuged, and resuspended in PBS. The cell suspension was divided into five tubes and spun down. Each cell pellet was flash frozen. To assay for viral proteins, one pellet, containing 2 × 10^6 cells, was resuspended in 40 µl of SDS sample buffer.

Samples were sonicated for 30 s and heated to 100°C for 5 min. 40 µl was loaded per lane of a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotted for 30 min at 15 V using a Bio-Rad Transblot semidry transfer cell. The blots were blocked with 5% nonfat dry milk for 1 h and incubated for 1–2 h with human, rabbit, or murine antibodies (diluted in 5% nonfat dry milk) to EBV lytic cycle proteins. The blots were washed twice in Tris-saline (TS) (10 mM Tris, pH 7.5, 200 mM NaCl, 5% Tween 20), incubated for 1–2 h with secondary antibodies (diluted in 5% nonfat dry milk) appropriate for the species, and washed twice in TS. To detect immunoreactive bands, blots were incubated with 1 µCi of ^125^I-protein A (Amersham Biosciences) in nonfat dry milk for 1 h and washed twice. The blots were exposed overnight with intensifying screens to Eastman Kodak Co. XAR-5 film at −70°C.

**Transmission Electron Microscopy—** 2089 cells were transfected with WT gZ-CMV and Z(R183E)-CMV plasmids using DMRIE-C reagent (Invitrogen). Samples were fixed in 2% paraformaldehyde for 30 min at 4°C, rinsed in PBS, resuspended in 10% gelatin, chilled, then trimmed to smaller blocks, and placed in cryoprotectant (2.3 M sucrose) overnight at 4°C. Blocks were transferred to aluminum pins and frozen rapidly in liquid nitrogen. Frozen blocks were trimmed on a Leica cryo-EM UC6 UltraCut, and 75-nm-thick sections were collected using the method of Tokuyasu (37). Sections were placed on a nickel Formvar/carbon-coated grid and floated in a dish of PBS for immunolabeling. Grids were placed section side down on drops of 0.1 M ammonium chloride for 10 min to quench untreated
RESULTS

Intracellular Distribution of ZEBRA Mutants That Do Not Bind DNA—We investigated the intracellular localization of 10 point mutants within the basic DNA-binding region of ZEBRA that abolished the ability of the protein to bind DNA (Fig. 1). These included seven mutants, K178D, K178E, R179E, R183E, R190E, K194D, and K194E, in which a basic aa was replaced by an acidic aa. These mutants were selected for study because 11 of the 19 non-DNA-binding mutants generated by Heston et al. (30) had replaced a basic aa with an acidic aa. The other three mutants were N182K in which a neutral aa was replaced by a basic aa, N182E in which a neutral aa was replaced by an acidic aa, and S186E in which the well studied serine at position 186, required for BRLF1 activation and absent from other AP-1-binding proteins (39), was changed to a glutamate. We transfected 2089 cells, which are 293 cells containing an EBV bacmid, with wild-type ZEBRA or each of the 10 non-DNA-binding mutants; the localization of these proteins was detected by immunofluorescence and confocal microscopy.

Wild-type (WT) ZEBRA was distributed diffusely throughout the nucleus (Fig. 1i) (31). The distribution of WT ZEBRA was smooth and uniform; ZEBRA was often concentrated at the nuclear periphery. Subregions devoid of ZEBRA correspond to nucleoli (31). With the exception of S186E, each point mutation resulted in a distinct intranuclear distribution that differed from the diffuse and relatively uniform distribution of WT ZEBRA (supplemental Table S1). The N182K, K194D, and K194E mutations produced a disordered, lumpy distribution in which ZEBRA protein was randomly scattered throughout the nucleus (Fig. 1, v, x, and xii). The K178E and N182E mutations produced a speckled distribution of ZEBRA protein (Fig. 1, iii and vi). The S186E mutation resulted in a diffuse and relatively even distribution (Fig. 1vii) that resembled WT ZEBRA. These studies showed that the failure of a mutant ZEBRA protein to bind DNA was not by itself associated with a distinct distribution phenotype.

The Z(K178D), Z(R179E), Z(R183E), and Z(R190E) mutants produced a distribution pattern that was different from that of WT ZEBRA or any mutant that had been studied previously. These mutants localized to two types of structures within the nucleus: numerous punctate foci of variable size and number (Fig. 2A, ii) and to fewer, relatively large, hollow, ring-shaped structures (Fig. 1, ii, iv, vii, and ix, blue arrows). The ring-shaped structures varied widely in size with diameters ranging from ~500 nm to 5 μm (Fig. 2B) and usually numbered fewer than eight per cell. Cells with larger rings contained fewer rings and fewer punctate foci.

Z(R183E) Induced Similar Structures in 293 Cells—We selected Z(R183E) as a prototype mutant for many further studies of the induction of the ring-shaped nuclear structures. Although WT ZEBRA was diffusely distributed in 293 cells that contain no EBV (31), transfection of the Z(R183E) mutant into these cells produced punctate foci and large ring-shaped inclusions similar to those seen in 2089 cells (Fig. 2A). This result indicated that the single Arg to Glu substitution in the basic domain of ZEBRA was sufficient to induce formation of the observed structures in the absence of any additional viral fac-
tors. However, the ring-shaped structures were more frequently observed in 2089 cells than in 293 cells (Fig. 2B). Approximately 43% of 2089 cells that expressed Z(R183E) contained ring-shaped structures (large, medium, and small rings); among 293 cells that expressed the Z(R183E) mutant, ~18% contained ring-shaped structures (p < 0.05). Taken together, the data indicate that the Arg to Glu mutation itself was sufficient to generate the mutant phenotype; however, additional viral or cellular factors that are present in 2089 cells may facilitate or enhance the generation of the ring-shaped structures.

Z(K178D), Z(R179E), Z(R183E), and Z(R190E) Mutants Are Partially Localized in Cytoplasm—Unlike WT ZEBRA or other ZEBRA mutants that localize exclusively to the nucleus, large amounts of Z(K178D), Z(R179E), Z(R183E), and Z(R190E) mutant proteins also localized to the cytoplasm as shown by co-staining for lamin B, which delineates the nuclear periphery (Fig. 2C, i–vi). In the cytoplasm, mutant ZEBRA protein was diffusely distributed and did not form punctate or ring-shaped structures. Cytoplasmic accumulation of Z(R183E) was also confirmed by Western blot analysis of transfected cell lysates (supplemental Fig. S1A). Unfractionated whole cell extracts contained nearly equal quantities of WT, S186A, and R183E forms of ZEBRA (supplemental Fig. S1B). Nucleolin and tubulin, which were used as markers for the separation of nuclear and cytoplasmic fractions, respectively, showed satisfactory separation of nuclear and cytoplasmic components (supplemental Fig. S1A). In cells transfected with empty vector (CMV), WT ZEBRA, or Z(S186A), ZEBRA protein was not detected in the cytoplasmic fraction, whereas a large amount of Z(R183E) was present in the cytoplasmic fraction.

Immunoelectron Microscopy of WT ZEBRA and Mutant Z(R183E)—We used transmission electron microscopy to examine the ultrastructure of the ring-shaped inclusions induced by Z(R183E) (Fig. 3). 2089 cells were transfected with empty vector (Fig. 3, i and ii), with WT ZEBRA (Fig. 3, iii and iv), or with Z(R183E) (Fig. 3, v and vi) and processed for immunogold labeling with an antibody to ZEBRA. Immunogold labeling was more intense in the nucleus of cells transfected with WT ZEBRA than in cells transfected with control plasmid. In cells transfected with WT ZEBRA, the gold particles were distributed throughout the intranuclear space but were concentrated at the more electron-dense heterochromatin. Prominent electron-dense inclusions within the nucleus were only observed in cells transfected with Z(R183E). These inclusions were circular or ring-shaped, and they consisted of a homogeneous granular material not surrounded by lipid membranes. Gold particles were heavily concentrated in the spherical intranuclear inclusions (Fig. 3v). When a hollow core was visible, the gold particles were distributed along the periphery of the ring-shaped inclusion but not in the interior (Fig. 3vi). The distributions of WT and Z(R183E) mutant ZEBRA protein

![FIGURE 1. Single amino acid changes in basic DNA-binding region of ZEBRA that abolish ability of protein to bind DNA also change its intracellular localization. 2089 cells were transfected with expression vectors for WT ZEBRA (i) or mutant ZEBRA proteins Z(K178D) (ii), Z(K178E) (iii), Z(R179E) (iv), Z(N182E) (v), Z(N182K) (vi), Z(R183E) (vii), Z(S186E) (viii), Z(R190E) (ix), Z(K194D) (x), and Z(K194E) (xi). Cells were fixed and then stained with antibody specific for ZEBRA and secondary antibodies conjugated with Texas Red or Rhodamine Red X. Digital images were acquired by confocal microscopy. Blue arrows indicate the ring-shaped structures induced by the Z(K178D), Z(R179E), Z(R183E), and Z(R190E) mutants.](https://www.jbc.org/content/286/11/9752)
observed by electron microscopy confirmed the distribution observed by immunofluorescence.

**Hollow Intranuclear Structures Induced by Z(R183E) Are Spherical in Shape**—The confocal images of ring-shaped structures induced by Z(R183E) suggested several possible three-dimensional progenitor shapes, including a cylinder oriented along the z axis, a hollow ovoid, and a hollow sphere. To determine the three-dimensional shape of the R183E-induced hollow structures, a Z-stack series of regularly spaced images were taken of a cell containing a large R183E-induced hollow structure, and a three-dimensional reconstruction was generated. Rotation of the three-dimensional image showed the hollow structure to be spherical in shape. In supplemental Fig. S2, the three-dimensional reconstruction was rotated 120° and 240° about the x axis, and the hollow body was shown to be circular throughout the rotation. This result indicates a spherical shape.
Had the object been ovoid or tubular in shape, progressive rotation about the \( x \) axis would have shown an elliptical or rectangular outline, respectively.

*Structures Induced by Z(R183E) Contain PML Protein and Chaperone HSP70 Protein, Two Major Components of Nuclear Aggresomes*—One possible explanation for the structures observed in cells transfected with the Z(R183E) mutant is that substitution of a positively charged arginine with a negatively charged glutamate may have prevented the proper folding of ZEBRA, leading to protein aggregation and subsequently to induction of aggresome formation. To test this hypothesis, we studied the expression and localization of two identifying constituents of nuclear aggresomes, PML protein and HSP70 protein, in cells transfected with wild type or Z(R183E) mutant.

In 2089 cells transfected with empty vector, PML protein localized to one to five small discrete punctate foci within each nucleus (Fig. 4A, i–iv). This distribution matches the localization of PML seen in most cell lines (40–42). During the lytic phase of EBV, PML protein has been reported to disperse from PML bodies (40). In agreement with this result, transfection of WT ZEBRA into 2089 cells and induction of the lytic phase resulted in the disappearance of PML protein in cells containing ZEBRA (Fig. 4A, v–viii). However, in cells transfected with Z(R183E), PML protein was localized to the interior “hollow” region of the spherical structures (Fig. 4A, ix–xi). A three-dimensional Z-stack reconstruction showed that PML protein within the spheres induced by mutant ZEBRA had the shape of a rounded globular body (data not shown). The globular body of PML in the interior of the putative aggresomes was surrounded by a shell of mutant Z(R183E) protein. In cells containing only the small punctate foci of Z(R183E) protein and no hollow aggresomes, there was very little if any redistribution of PML.

![Figure 3. Ultrastructure of inclusion bodies induced by Z(R183E) seen by immunoelectron microscopy.](image-url)
protein (Fig. 4A, xii–xiv). This result indicated that the redistribution of PML protein correlates with the appearance of the larger “hollowed” aggresomes.

In cells transfected with Z(R183E), HSP70 localized to the interior region of the spherical structures (Fig. 4B, vii–xv). HSP70 within these spheres was distributed as a rounded globular body, was diffusely present throughout the interior volume, and was more evenly dispersed than was PML protein. HSP70 staining was not observed within the nucleus of 2089 cells transfected with empty vector (Fig. 4B, i–iii). In cells transfected with wild-type ZEBRA, there was a slight increase in HSP70 seen diffusely throughout the nuclear and cytoplasmic space in cells positive for ZEBRA (Fig. 4B, iv–vi; three ZEBRA-positive cells shown). The presence of both PML protein and HSP70 within the spherical structures induced by mutant ZEBRA indicates that these spherical structures are aggresomes.

**Nuclear Aggresomes Induced by Z(R183E) Recruit HDAC6**—HDAC6, a class II histone deacetylase, actively localizes to the cytoplasm and functions as the primary deacetylase of α-tubulin (43). HDAC6 is a component of cytoplasmic aggresomes and plays an essential role in their formation (10, 44, 45). By simul-

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**FIGURE 4.** PML protein, HSP70, and HDAC6 are recruited to cells containing Z(R183E)-induced nuclear aggresomes. A, PML protein redistributed from ND10 to a globular IB located within the interior volume of the aggresomes. 2089 cells were transfected with empty vector (i–iv), WT ZEBRA (v–viii), or Z(R183E) (ix–xiv). Cells were fixed and doubly stained for PML protein and ZEBRA. B, the chaperone HSP70 accumulated within the interior of spherical structures composed of Z(R183E) protein. 2089 cells were transfected with empty vector (i–iii), WT ZEBRA (iv–vi), or Z(R183E) mutant ZEBRA (vii–xv). Cells were fixed and stained for HSP70 protein and ZEBRA. C, Z(R183E)-induced nuclear aggresomes contain HDAC6. 2089 cells were transfected empty vector (i–iii), WT ZEBRA (iv–ix), or Z(R183E) (x–xv), then fixed, and stained for EA-D (i, iii, iv, vi, vii, and ix), ZEBRA (x, xi, xii, and xv), and HDAC6 (v, vii, x, and xii). Transm., transmitted.
taneous interaction with polyubiquitin and dynein motors, HDAC6 recruits ubiquitinated misfolded proteins to dynein for transport to cytoplasmic aggresomes. Recruitment of HDAC6 to nuclear aggresomes or a role for HDAC6 in formation of nuclear aggresomes has not yet been reported. We asked whether HDAC6 was recruited to nuclear aggresomes induced by mutant ZEBRA. In 2089 cells transfected with empty (CMV) vector (Fig. 4C, i–iii) or induced to enter the lytic phase by transfection with WT ZEBRA (Fig. 4C, iv–ix), HDAC6 localized exclusively to the cytoplasm. In cells transfected with Z(R183E), HDAC6 was strongly recruited to nuclear aggresomes (Fig. 4C, x–xv). In most cells, HDAC6 filled the internal volume of the aggresome (Fig. 4C, x–xii); however, in some cells, HDAC6 appeared to localize to the aggresomal periphery (not shown). Although recruitment of HDAC6 to nuclear aggresomes was efficient, a significant amount remained localized to the cytoplasm in cells with nuclear aggresomes.

Aggresomes Induced by Z(R183E) Recruit SC35 but Not Nucleolin—Aggresomes selectively recruit properly folded wild-type proteins (46). We examined the ability of Z(R183E)-induced aggresomes to recruit and sequester two cellular proteins, SC35 and nucleolin, whose specific distribution within cell nuclei is well documented (31, 47–49). SC35 is a spliceosome assembly factor that localizes to multiple discrete, irregularly shaped subnuclear organelles alternatively called “SC35 domains,” “splicing speckles,” or “interchromatin granules” (47, 50).

In 2089 cells transfected with CMV empty vector, SC35 was localized to 10–20 discrete, irregularly shaped intranuclear foci (supplemental Fig. S3, i–iv). In 2089 cells transfected with WT ZEBRA (Z) and induced to enter the lytic phase of EBV, SC35 was redistributed into foci that were significantly larger, brighter, more round, more distinct, and fewer in number than seen in cells transfected with empty vector (supplemental Fig. S3, v–viii). This same redistribution of SC35 has been described in uninfected Vero cells transfected with the EBV Mta protein (48).

SC35 was relocalized exclusively to aggresomes in 2089 cells transfected with WT ZEBRA (Z) and induced to enter the lytic phase of EBV (Fig. 5). SC35 staining within the interior of large hollow aggresomes was invariably bright. In contrast to PML or HSP70 proteins, which localized exclusively to the interior, or mutant ZEBRA protein, which localized exclusively to the periphery of aggresomes, SC35 localized both to the periphery and to the interior regions.

In 2089 cells transfected with empty vector, nucleolin appeared as two to five prominent spherical foci within the nucleus that were characteristic of nucleoli (supplemental Fig. S4, i–iv). In cells transfected with WT ZEBRA and induced to enter the lytic phase, nucleolin localized to the regions of the nucleus spared of ZEBRA (supplemental Fig. S4, v–viii) (31). Lytic induction also caused redistribution of nucleolin; rather than being confined to a discrete dot, nucleolin within cells expressing ZEBRA was visibly dispersed. Dispersal of nucleolin also occurs during lytic infection of HSV-1 and is mediated by the UL24 protein (51, 52). In cells transfected with Z(R183E), nucleolin was not recruited to aggresomes (supplemental Fig. S4, ix–xii). Like WT ZEBRA, Z(R183E) caused nucleolin to disperse but did not co-localize with nucleolin.
FLAG-tagged ZEBRA, Rta, and BMLF1 Are Recruited to Z(R183E)-induced Aggresomes; However, EA-D Is Not—The induction of nuclear aggresome formation by Z(R183E) and its recruitment to aggresomes raised the question of whether properly folded WT ZEBRA would also be recruited to aggresomes. A FLAG-tagged version of WT ZEBRA (FLAG-WT-ZEBRA) allowed simultaneous monitoring of both mutant Z(R183E) and the FLAG-tagged version of WT ZEBRA within a single cell.

When transfected alone into 2089 cells, FLAG-WT-ZEBRA was present in a distribution similar to that of non-tagged WT ZEBRA. FLAG-WT-ZEBRA was diffusely distributed throughout the nucleus but spared nucleolar regions (Fig. 5, i–iii). FLAG-WT-ZEBRA also induced the lytic phase of EBV. EA-D was produced and localized in mature globular viral replication compartments (data not shown). When FLAG-WT-ZEBRA was co-transfected with Z(R183E), in all cells that contained nuclear aggresomes, FLAG-WT-ZEBRA was present in the aggresomes (Fig. 5, iv–vi). FLAG-WT-ZEBRA was recruited strongly to both large hollow aggresomes and to smaller punctate aggregates (Fig. 5, vii–ix). FLAG-WT-ZEBRA co-localized with Z(R183E) at the periphery of larger aggresomes and was absent from the interior volume. Whereas Z(R183E) localized only to the periphery, FLAG-WT-ZEBRA was partially included in the interior of aggresomes (not shown). The recruitment of FLAG-WT-ZEBRA to aggresomes was robust; its recruitment to aggresomes and removal from the surrounding nuclear space was more complete than the recruitment of Z(R183E) (Fig. 5, compare iv and v). Another obvious difference in distribution between Z(R183E) and FLAG-WT-ZEBRA was the absence of the WT protein in the cytoplasm.

We examined the localization of three lytic phase viral proteins, EA-D, Rta, and BMLF1, in cells containing aggresomes induced by ZEBRA mutants (Fig. 6). Because Z(R183E) does not induce the lytic phase, 2089 cells were co-transfected with Z(R183E) and Rta, which allowed simultaneous examination of aggresome formation by Z(R183E) and induction of the lytic phase by FLAG-WT-ZEBRA. In cells containing nuclear aggresomes, EA-D was never recruited to aggresomes. EA-D remained diffusely distributed and was absent from regions containing aggresomes (Fig. 6, i–iii). In 2089 cells co-transfected with Rta and Z(R183E), Rta protein was recruited to aggresomes (Fig. 6, iv–vi). Like ZEBRA, Rta localized to the periphery of aggresomes and was often
excluded from the center, giving a hollow appearance. Unlike ZEBRA, however, recruitment of Rta to aggresomes was relatively weak, and much of the protein remained diffusely distributed in the nucleus. 2089 cells co-transfected with FLAG-WT-Z and R179E and then stained for FLAG and BMLF1 also showed recruitment of BMLF1 to nuclear aggresomes (Fig. 6, vii–ix). Thus, three non-mutant viral proteins were sequestered in aggresomes induced by Z(R183E) and Z(R179E) mutant ZEBRA.

Z(R183E) and Z(R179E) Exert Dominant Negative Effects on Lytic Replication of EBV—Because aggresomes induced by Z(R183E) and Z(R179E) recruit, sequester, and possibly degrade WT viral proteins such as ZEBRA, Rta, and BMLF1, we investigated whether the aggresome-inducing mutants of ZEBRA would inhibit viral replication. To test this hypothesis, BZKO cells were lytically induced by transfection of constant amounts of WT ZEBRA (WT gZ) plasmids and co-transfection with a range of concentrations of Z(R183E) or Z(R179E) plasmid. The amount of WT gZ DNA plasmid used for these experiments was determined by titration of WT gZ and immunoblot visualization of two EBV late lytic proteins, BFRF3 (FR3) and BLRF2 (LR2) (Fig. 7A). 100 ng of WT gZ induced a signal of these two proteins that was 16- and 50-fold above background levels (Fig. 7, A, B, and C). Co-transfection of increasing amounts of Z(R183E) inhibited FR3 late protein expression by more than 90% at ratios of mutant to WT plasmid equal to or greater than 10:1 and almost completely inhibited LR2 late protein expression at a ratio of 5:1 or more (Fig. 7B). Under similar conditions, mutant Z(R179E) inhibited FR3 expression at ratios equal to or greater than 5:1 relative to wild type (Fig. 7C). In the same experiment, Z(R179E) was less potent at inhibiting expression of the early viral lytic gene marker EA-D. At a 5:1 ratio, Z(R179E) inhibited EA-D expression by 31%. Complete inhibition of expression of EA-D was not evident until the Z(R179E) mutant to wild-type ratio was 20:1.

The effect of one aggresome-inducing mutant, Z(R183E), on lytic viral DNA synthesis was assessed by Southern blotting (Fig. 7D). BZKO cells were co-transfected with constant amounts of WT gZ and increasing amounts of Z(R183E). Cellular DNA was extracted and digested with BamHI, and blots
were probed with a radiolabeled XhoI subfragment located adjacent to the termini of EBV DNA and with the EBV large internal repeat (BamHI W). The ladder of DNA fragments, representative of lytic viral DNA replication, was reduced at a ratio of 5:1 of mutant to wild-type input DNA and was abolished at Z(R183E) to WT plasmid ratios of 10:1 and 20:1. This inhibitory ratio was similar to that which eliminated FR3 expression (Fig. 7B). Based on the BamHI W signal, there was a progressive decrease in the amount of EB viral DNA beginning at a 1:1 ratio of mutant to WT bzlf1 input plasmid (Fig. 7D). Thus, whether assessed by the abundance of EBV late protein or the abundance of lytic EBV DNA, two ZEBRA mutants that induce the formation of nuclear aggresomes inhibit EBV replication.

**DISCUSSION**

**Single Amino Acid Substitutions in Viral Protein ZEBRA Induce Formation of Nuclear Aggresomes**—In studying changes to the intracellular localization of ZEBRA after introduction of mutations that prevent binding of ZEBRA to DNA, we observed that specific missense mutations in the DNA-binding region, K178D, R179E, R183E, and R187E, induced the formation of prominent spherical IBs within the nucleus. Subsequent investigation into the nature of these IBs identified them as nuclear aggresomes, similar to those tightly linked to polyQ neurodegenerative diseases and also shown to be induced by two other non-polyQ mutant GFP fusion proteins. The aggresomes induced by mutant ZEBRA existed as multiple discrete foci or circular ring-shaped structures in the nucleus in contrast to the single large perinuclear IB seen with cytoplasmic aggresomes. The formation of nuclear aggresomes is invariably accompanied by redistribution and recruitment of PML protein and recruitment of HSP chaperones. Induction of aggresomes by mutant ZEBRA likewise coincided with redistribution of PML and recruitment of PML and HSP70. The mutant ZEBRA proteins were strongly recruited to the aggresomes but localized to the periphery, forming an outer layer surrounding the globular core, an organization commonly seen in other nuclear aggresomes. Aggresomes induced by mutant ZEBRA also possessed the characteristic ability to selectively recruit additional non-mutated cellular and viral proteins (i.e. SC35, HDAC6, wild-type ZEBRA, Rta, and BMLF1). Transfection of the mutant ZEBRA proteins into cells lacking EB virus was sufficient to induce formation of aggresomes albeit to a lesser extent, indicating that the mutant viral proteins were directly responsible for activating the nuclear aggresome response.

**Comparing Induction of Nuclear Aggresomes by Mutant ZEBRA and by Mutant Human Cytomegalovirus**—Formation of nuclear aggresomes has been described during infection with a mutant version of a related herpesvirus, human cytomegalovirus (HCMV), lacking the viral UL97 kinase (46). The aggresomes induced by HCMV shared several properties with the mutant ZEBRA-induced aggresomes described here. Both types of aggresomes contained PML and HSP chaperones. Both types of aggresomes sequestered cellular proteins and several non-mutated viral proteins. Induction of each aggresome coincided with an inhibition of viral replication. The sequestration of HCMV proteins by virally induced aggresomes was proposed by Prichard et al. (46) as evidence that induction of aggresomes may constitute an innate cellular antiviral response against viral replication. Our results showing that EBV proteins are sequestered in nuclear aggresomes and that EBV replication is inhibited in cells induced to form aggresomes support this hypothesis.

Despite these similarities, however, significant differences between HCMV and EBV are likely to exist regarding their interactions with host aggresomal mechanisms. The ability of UL97 kinase to prevent aggresome formation during HCMV infection and to inhibit the aggregation of pp65 in a kinase-dependent manner indicates a role for UL97 as a virally encoded means of evading the nuclear aggresome response (53). There is yet no evidence that an analogous factor exists for EBV. Moreover, it is unlikely that BGLF4 kinase, the EBV homolog of UL97, acts to suppress nuclear aggresome formation because co-transfection of BGLF4 kinase with Z(R183E) did not reduce the efficiency of aggresome formation (data not shown). Although UL97 has been identified as a suppressor of the aggresomal response during HCMV infection, the identity of the viral protein(s) that directly induced the formation of nuclear aggresomes during HCMV infection in the absence of UL97 is not known. Nor is it known how loss of UL97 kinase activity may elicit the aggresome response. To our knowledge, the studies described here are the first report of a mutant viral protein that directly and robustly induced the formation of nuclear aggresomes.

**Nuclear Aggresomes Induced by Mutant ZEBRA Have Distinct Spatial Organization**—Nuclear aggresomes are dynamic structures that rapidly and continually exchange components with the surrounding nuclear space. They possess complex internal substructures that undergo extensive repositioning as aggresomes increase in size (15). The larger aggresomes induced by mutant ZEBRA proteins were organized into at least two distinct layers. PML and HSP70 proteins resided as a globular mass in the interior of the aggresome and were surrounded by mutant ZEBRA protein at the aggresomal periphery (Fig. 4, A, ix–xi, and B, vii–xv). SC35 protein appeared to localize to the aggresomal periphery and to the interior. A similar organization has been described for nuclear aggresomes induced by other mutant proteins. For example, truncated and tagged ataxin-3 and ataxin-1 proteins localized to the aggresomal periphery in a ring-shaped pattern in each of their respective aggresomes (12). In nuclear aggresomes induced by mutant ataxin-3 or by GFP-170*, PML existed as a globular mass in the aggresomal interior (12, 15). Nuclear aggresomes induced by truncated ataxin-3 recruited SC35 protein. In contrast, some nuclear aggresomes showed different patterns of organization. In nuclear aggresomes induced by full-length myc-tagged ataxin-3 or by UV irradiation, PML was found in a ring-shaped localization at the aggresomal periphery (12, 20). Truncated ataxin-2 and GFP-170* were distributed as a solid mass within their respective aggresomes. Nuclear aggresomes induced by full-length ataxin-3 or by GFP-170* did not recruit SC35 (12, 15). As has been proposed, the differences in organization and composition seen among various nuclear aggresomes may reflect variations in the cellular aggresomal response to differences in the aggregating proteins (12). The particular composition and structure of mutant ZEBRA-induced aggresomes,
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compared with nuclear aggresomes induced by other mutant proteins, may therefore be regulated by the nature of the mutation itself.

Mechanisms by Which ZEBRA Mutants Might Induce Formation of Aggresomes—Although cytoplasmic aggresomes are induced by a range of differing mutations, nuclear aggresomes have heretofore been shown to be induced only by expansions of polyQ tracts and by the two GFP fusion non-polyQ constructs GFP-170° and REDA87–113GF. We show that nuclear aggresomes can be efficiently induced by single missense mutations within the ZEBRA DNA-binding domain. Of 17 single amino acid substitutions made to the DNA-binding region of ZEBRA, five resulted in the efficient induction of nuclear aggresomes (supplemental Table S1). These five mutations, K178D, R179E, R183E, R187E, and R190E, share two common traits: each replaced a basic residue with an acidic one, and each abolished the ability of ZEBRA to bind DNA. Four of these five were arginine to glutamate substitutions. Thus far, every arginine to glutamate substitution in the basic domain of ZEBRA has resulted in aggresome induction. Expression of the Z(K178D) mutant resulted in aggresome formation; however, Z(K178E), Z(K194D), and Z(K194E) mutants did not elicit the nuclear aggresome response. Glutamate substitutions at two other positions, Z(N182E) and Z(S186E), also abolished binding to a ZIIIB DNA site but did not induce aggresome formation (Fig. 1). Taken together, these data indicate that a combination of factors, including a change in charge and a loss of DNA binding, may be involved in the induction of nuclear aggresomes.

Predicted Effects of Mutations on Structure of ZEBRA—At least three hypotheses can be proposed to account for the mechanism by which the basic to acidic aa substitutions that alter the conformation of ZEBRA might trigger the aggresome response. (i) The substitutions may affect the nature of the interactions of ZEBRA with DNA. (ii) They may alter amino acid contacts within the α helix. (iii) They may disrupt homodimer formation. These hypotheses are informed by the single published crystal structure of ZEBRA, namely Z(S186A/C189S) bound to an AP-1 site from the BMLF1 promoter (27). Based on this crystal structure, we used SWISS-PDB and PyMol to predict the effects of specific amino acid substitutions on interactions with DNA (supplemental Table S2).

In the ZEBRA crystal structure, amino acids Arg-179, Arg-183, and Arg-187 contact the phosphate backbone, and Arg-190 contacts a base at the AP-1 site (27). Glutamate substitutions at these positions are predicted to obliterate interactions with DNA at the AP-1 site (supplemental Table S2). The crystal structure also shows that Asn-182 and Ser-186 (substituted with Ala) contact DNA bases. However, glutamate substitutions at positions Asn-182 and Ser-186 are not predicted to obliterate contact with either the phosphate back bone or DNA bases. Contact with DNA at specific sites within the ZEBRA DNA-binding domain may be sufficient to prevent protein misfolding and subsequent aggresome formation (supplemental Table S2). Further structural studies would be needed to determine whether any of these changes alter the configuration of the continuous α helix. It is likely that a cellular mechanism exists to detect specific alterations in protein folding that generate protein aggregates. These would be recognized by the cell and targeted for association with PML for transport to nuclear aggresomes. The presence of the chaperone HSP70 in mutant ZEBRA-induced aggresomes supports the concept that specific types of misfolding trigger the aggresome response.

Perturbations of interactions between the DNA-binding domain of ZEBRA and DNA as a result of single amino acid substitutions at specific sites occupied by arginines may promote an energetically favorable, aggregation-prone conformation that would otherwise not exist when ZEBRA is bound to DNA. Structurally, ZEBRA deviates from canonical bZIP proteins as it possesses a C-terminal tail that runs antiparallel to its coiled coil domain and is separated from its bZIP domain by a hairpin turn (27). This unique feature of ZEBRA lends itself to domain swapping, a well described structural mechanism for protein aggregation in proteins that harbor two independently folded domains connected by a loop (54–56). This C-terminal region of ZEBRA has been reported to interact with the zipper domain nearly as far as the basic domain (57). Perturbations in the stability of the DNA-binding domain as a result of amino acid substitutions may be transmitted to other regions of the ZEBRA homodimer, including the C-terminal regions. Consequently, the antiparallel C-terminal regions may occasionally be exposed and thus provide an interface for interaction with other ZEBRA monomers. This would then lead to a nucleating structure for aggregation of homo-oligomeric complexes. The overexpression of ZEBRA mutants with disordered DNA-binding regions may increase the likelihood of domain swapping, leading to aggregation.

The fact that aggresome formation in our study is compartmentalized to the nucleus raises the possibility that within the nucleus the structure of ZEBRA is more sensitive to perturbation than in the cytoplasm. Two cysteines at position Cys-222 in each ZEBRA monomer, spaced 3.9 Å apart (too far apart to form a disulfide bond), positioned within the C-terminal region of ZEBRA are functionally significant for the redox sensitivity of ZEBRA DNA binding (27, 58). These cysteines may be involved in mediating aggregation. They may be impacted differently by the redox environment in the nucleus versus the cytoplasm. It is not clear why basic to acidic mutations within the ZEBRA basic domain involving arginines but not lysines consistently lead to aggresome formation. Inspection of the basic domains of ZEBRA homologs in other non-human primate herpesviruses reveals conserved arginines coinciding with positions Arg-179, Arg-183, Arg-187, and Arg-190 of ZEBRA. Positions occupied by lysines within the DNA-binding domain of ZEBRA are in some cases substituted for arginines at various positions in these ZEBRA homologs. Therefore residues Arg-179, Arg-183, Arg-187, and Arg-190 of ZEBRA must each play a crucial role in the maintenance of overall stability of the ZEBRA protein.

Protein-Protein Interactions between ZEBRA and PolyQ Proteins—Protein-protein interactions with polyQ-containing transcriptional activators may indirectly induce the aggresome response. Although ZEBRA does not contain a polyQ expansion domain, it is known to interact with at least two polyQ-containing proteins that are also implicated in polyQ diseases.
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ZEBRA functionally interacts with CREB-binding protein, a protein that contains a polyglutamine tract (59). CREB-binding protein is sequestered in nuclear inclusions formed by mutants of the polyQ protein androgen receptor, which is implicated in spinal and bulbar muscular atrophy (60). The pathogenic domain of mutant Huntington disease protein (htex1p) containing expanded polyQ repeats has also been found to interact with CREB-binding protein (17). ZEBRA functionally interacts with TATA box-binding protein, another polyQ-containing protein implicated in spinocerebellar ataxia 17 (61–63). Aggresome formation by the ZEBRA mutants reported in this study may be mediated by interactions with the same polyQ proteins that interact with WT ZEBRA. In the event that interactions with CREB-binding protein and TATA box-binding protein, which reside in the nucleus, are important for aggresome formation, this would explain why, in our case, aggresome formation is restricted to the nucleus. Understanding how interactions between ZEBRA and polyQ-containing proteins are involved in aggresome formation may provide insight on the pathogenesis of polyglutamine disorders.

In summary, we found that specific point mutations in the DNA-binding domain of a virally encoded transcription factor and replication protein result in highly efficient induction of nuclear aggresomes that recruit other non-mutant cellular and viral proteins. The recruitment of viral proteins is associated with inhibition of viral replication. The specificity and simplicity of this aggresome-inducing alteration may aid in the future study and elucidation of the mechanisms involved in the intranuclear aggresome response. These observations also raise the possibility that certain mutant viral proteins, by triggering the aggresome response, might play a role in initiating disease processes that are the result of protein misfolding and the toxic accumulation of mutant protein aggregates.

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