Crystal Structure of Cytomegalovirus IE1 Protein Reveals Targeting of TRIM Family Member PML via Coiled-Coil Interactions

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Abstract

PML nuclear bodies (PML-NBs) are enigmatic structures of the cell nucleus that act as key mediators of intrinsic immunity against viral pathogens. PML itself is a member of the E3-ligase TRIM family of proteins that regulates a variety of innate immune signaling pathways. Consequently, viruses have evolved effector proteins to modify PML-NBs; however, little is known concerning structure-function relationships of viral antagonists. The herpesvirus human cytomegalovirus (HCMV) expresses the abundant immediate-early protein IE1 that colocalizes with PML-NBs and induces their dispersal, which correlates with the antagonization of NB-mediated intrinsic immunity. Here, we delineate the molecular basis for this antagonization by presenting the first crystal structure for the evolutionary conserved primate cytomegalovirus IE1 proteins. We show that IE1 consists of a globular core (IE1CORE) flanked by intrinsically disordered regions. The 2.3 Å crystal structure of IE1CORE displays an all α-helical, femur-shaped fold, which lacks overall fold similarity with known protein structures, but shares secondary structure features recently observed in the coiled-coil domain of TRIM proteins. Yeast two-hybrid and coimmunoprecipitation experiments demonstrate that IE1CORE binds efficiently to the TRIM family member PML, and is able to induce PML deSUMOylation. Intriguingly, this results in the release of NB-associated proteins into the nucleoplasm, but not of PML itself. Importantly, we show that PML deSUMOylation by IE1CORE is sufficient to antagonize PML-NB-instituted intrinsic immunity. Moreover, co-immunoprecipitation experiments demonstrate that IE1CORE binds via the coiled-coil domain to PML and also interacts with TRIM5α. We propose that IE1CORE sequesters PML and possibly other TRIM family members via structural mimicry using an extended binding surface formed by the coiled-coil region. This mode of interaction might render the antagonizing activity less susceptible to mutational escape.

Introduction

Promyelocytic leukemia protein PML is the organizer of small nuclear matrix structures termed nuclear bodies (NBs) or nuclear domain 10 (ND10) [1]. PML, also named TRIM19, is a member of the tripartite motif (TRIM) family of proteins, which are characterized by the presence of RING, B-box and coiled-coil domains [2]. Recent studies showed that an unprecedented large number of TRIMs positively regulate innate immune signaling pathways by acting as E3-Ub ligases [3,4]. Additionally, a subgroup of TRIMs, including PML, was demonstrated to exhibit small ubiquitin related modifier (SUMO) E3 activity and PML itself is covalently conjugated to SUMO on three lysine residues [5,6]. This modification, which affects PML localization, stability and interaction with other partners, is critical for NB functions [7]. In response to stimuli, PML-NBs recruit a number of proteins implicated in different cellular processes such as DNA damage response, apoptosis, senescence and protein degradation [8,9]. Accumulating evidence implicates this subnuclear structure as an important component of intrinsic immunity against viruses from different families including herpes-, adeno-, polyoma, rhabdo- and retroviruses [10–12]. Unlike the innate and adaptive immunity, the intrinsic immune response is mediated by cellular restriction factors that are constitutively expressed and permanently active, even before a pathogen enters the cell. Other characteristics of intrinsic immune mechanisms are that they are saturable and subject to viral countermeasures [13]. Besides PML, a number of NB components, such as Sp100, hDaxx and ATRX, function as cellular restriction factors. Recent evidence suggests that NB proteins independently contribute to the repression of
Author Summary

Research of the last few years has revealed that microbial infections are not only controlled by innate and adaptive immune mechanisms, but also by cellular restriction factors, which give cells the capacity to resist pathogens. PML nuclear bodies (PML-NBs) are dot-like nuclear structures representing multiprotein complexes that consist of the PML protein, a member of the TRIM family of proteins, as well as a multitude of additional regulatory factors. PML-NB components act as a barrier against many viral infections; however, viral antagonistic proteins have evolved to modify PML-NBs, thus abrogating this cellular defense. Here, we delineate the molecular basis for antagonization by the immediate-early protein IE1 of the herpesvirus human cytomegalovirus. We present the first crystal structure for the evolutionarily conserved core domain (IE1CORE) of primate cytomegalovirus IE1, which exhibits a novel, unusual fold. IE1CORE modifies PML-NBs by releasing other PML-NB proteins into the nucleoplasm which is sufficient to antagonize intrinsic immunity. Importantly, IE1CORE shares secondary structure features with the coiled-coil domain (CC) of TRIM factors, and we demonstrate strong binding of IE1 to the PML-CC. We propose that IE1CORE sequesters PML and possibly other TRIM family members via an extended binding surface formed by the coiled-coil domain.

herpesvirus replication, raising the concept that individual NB components, rather than the PML-NB structure as a whole, restrict viral infections [14–20]. Consequently, various viruses have been shown to antagonize the intrinsic cellular defense via the modification of NB proteins. For instance, the herpes simplex virus type I immediate-early protein ICP0 has been described as a viral ubiquitin ligase with preferential substrate specificity for SUMO-modified isoforms of PML thus promoting the degradation of PML [21]. However, no structural information on this and many other NB-antagonistic proteins is available, yet.

Human cytomegalovirus (HCMV), a ubiquitous beta-herpesvirus causing serious disease in immunocompromised individuals, encodes an abundant immediate-early protein termed IE1 that modulates innate immune mechanisms as well as other cellular processes (reviewed in [22]). Although IE1 is a major player in the initiation of lytic HCMV infection and has been subject to extensive studies over the last decades, structural data on this protein are still limited. Four distinct regions have been identified within the 491 amino acid IE1 protein: a short N-terminal region that is required for nuclear import, a large core domain, an acidic region near the C-terminus that harbors a SUMOylation site and a 16 amino acid chromatin-tethering domain (CTD) at the extreme C-terminus [23–26]. Recent results have suggested that the acidic C-terminal region of IE1 is characterized by a lack of well-defined three-dimensional structure, but contains a binding motif for signal transducer and activator of transcription (STAT) proteins. This interaction site enables IE1 to compromise STAT-mediated interferon signaling, thereby interfering with a crucial branch of the innate immune system and promoting viral replication [27–29]. In addition to its effects on the innate immune system, IE1 is required to overcome the PML-NB-mediated intrinsic immunity that targets HCMV immediately upon infection. IE1 transiently co-localizes with PML-NBs during the first 2–4 hours after infection but subsequently induces disruption of these structures [30–32]. NB dispersal correlates with the functional activities of IE1 during infection and a PML knock-down efficiently compensates for IE1 in promoting replication of an IE1-deficient virus, establishing IE1 as an important antagonist of PML-mediated cellular repression of viral replication [15,16,33]. Studies on the mechanism of NB dispersal have demonstrated that IE1 induces the loss of the SUMOylated forms of PML, and also influences the SUMOylation state of Sp100 [34,35]. However, in contrast to ICP0, this neither requires proteasomal activity nor does IE1 affect the abundance of unmodified PML [24,35]. In further studies, a physical interaction between IE1 and PML, which requires the N-terminal TRIM region of PML, has been detected as prerequisite for the transient co-localization and subsequent disruption of PML-NB integrity. The interaction site for PML has been mapped to the large core region of IE1, since deletions or mutations affecting this domain abrogate PML binding and NB disruption [23,33,36]. However, it was noted in several reports that mutations in the core region often result in unstable IE1 proteins, so that the molecular basis for the IE1-PML interaction remains uncharacterized [37,30].

Here we report the crystal structure of the evolutionarily conserved globular core domain of primate cytomegalovirus IE1 proteins, determined to 2.3 Å resolution. Unexpectedly, the overall structure does not resemble any known protein fold, but exhibits an unusual all-α-helical, femur-like shape which shares secondary structure features recently observed in the coiled-coil domain of TRIM proteins. We show that this IE1CORE domain binds with high affinity to PML via the coiled-coil domain. This induces PML de-SUMOylation thus releasing the PML-associated factors hDaxx, Sp100 and ATRX, while PML accumulations itself are not dispersed. Since IE1CORE efficiently complements lytic replication of an IE1-deleted HCMV, we conclude that sequestration of PML via IE1CORE is sufficient for antagonization of NB-mediated intrinsic immunity. Thus, cytomegaloviruses may have evolved a distinct structural fold to effectively bind and neutralize an important cellular hub protein that exerts critical roles during the regulation of innate immune responses as well as the control of programmed cell death [6–10,12].

Results

IE1 consists of a globular core domain flanked by intrinsically disordered regions

In order to further clarify the mechanism of IE1-mediated PML antagonization, we investigated the molecular architecture of the IE1 proteins from human, chimpanzee and rhesus cytomegalovirus (h-, c- and rhIE1) (Figure S1). As previously proposed by Krauss et al. [28], in silico predictions using the web server IUPred [39] suggested that the N- and C-terminal regions of all IE1 proteins display consistently high intrinsic disorder propensities (Figure 1A). Based on these predictions as well as on sequence conservation and the characterization of protease-resistant IE1 subdomains, we generated truncated IE1 constructs covering the folded core (Figure 1B). Limited proteolysis of recombinant full-length hIE1 as well as of C- or N/C-terminally truncated hIE1 proteins confirmed the in silico predictions (Figure 1C). These studies revealed the existence of a stably folded IE1CORE domain of about 360 residues (Figure 1C, hIE1 20-382) that is flanked at the N- and C-termini by intrinsically disordered regions (IE1N-IDR and IE1C-IDR). Circular dichroism (CD) spectroscopy [40,41] was applied to investigate the secondary structure composition of the IE1 variants. All hIE1 proteins produced typical α-helical spectra with negative ellipticity above 200 nm and two distinct minima at 208 nm and 222 nm (Figure 1D and S2). The spectra of full-length hIE1 and hIE1CORE differed in the region below 210 nm. Calculation of the difference spectrum revealed strong random coil
characteristics thereby confirming the predicted predominately disordered nature of the terminal regions (Figure S2).

Sequence identities between 24 and 73% between h-, c- and rhIE1CORE domains suggest that the core domains share identical folds. Indeed, the CD spectra of the recombinant proteins hIE1CORE, cIE1CORE and rhIE1CORE match extremely well and indicate that all core domains consist mainly of \(\alpha\)-helical segments (Figure 1D).

Crystal structure and architecture of IE1

Crystallization trials with full-length hIE1 remained unsuccessful and, in case of the IE1CORE variants, yielded suitable crystals only for rhIE1CORE after chemical methylation of surface exposed lysine residues. The structure of rhIE1CORE was solved using experimental phases and refined to 2.3 Å resolution (\(R_{\text{work}} = 19.73\%\), \(R_{\text{free}} = 24.96\%\)) (Figures 2 and S1). The main chain of the model was traced between amino acids 41 and 393. Since no well-defined electron density was visible for residues preceding residue 41 or following residue 393, we conclude that the core domain spans at least amino acids 42 to 392 of rhIE1 corresponding to residues 27 to 399 of hIE1. RhIE1CORE consists of a total of 11 \(\alpha\)-helices (Figure 2 and S1). Helices H3 and H9 are unusually long and contain as many as 16 and 17 helical turns, respectively. RhIE1CORE adopts an elongated, fan-like shape with dimensions of 130×25×25 Å\(^3\).

The structure can be divided into three distinct regions, namely an N-terminal head region (rhIE1: residues 62–118, 236–283; hIE1 residues 46–103, 221–267) and a C-terminal head region (rhIE1 residues 151–207, 315–393; hIE1 residues 136–192, 300–380) interconnected by a stalk region (rhIE1 residues 41–61, 119–150, 208–235, 284–314; hIE1 residues 27–45, 104–135, 193–220, 268–299) (Figure 2). The stalk consists of an uncommon right-handed three-helix coiled-coil (\(\alpha\)-helices H3, H6 and H9) with the N-terminal helix H1 added to one side of the three-helix bundle (Figure 2). The right-handed pairing of the helices goes in hand with the presence of hendecad repeats in the sequences of these helices [42]. In these repeats of 11 residues (numbered alphabetically \(abcdefghijk\)) the hydrophobic amino acids at positions \(a\), \(d\) and \(h\) are interspaced by 2 (\(bc\)), 3 (\(efg\)) and 3 (\(ijk\)) amino acids of predominantly polar nature (Figure S1). In contrast, the patterning of hydrophobic residues within the head regions of rhIE1CORE frequently resembles that observed in heptad repeats (residue labeling \(abcdefg\)). Here the hydrophobic amino acids at positions \(a\) and \(d\) are interspaced by 2 (\(bc\)) and 3 (\(efg\)) polar residues, and the interdigitation of the \(a\) and \(d\) residues from neighboring helices gives rise to more commonly observed left handed coiled-coil supersecondary structure elements [42]. Hence, both head regions display left-handed coiled-coils. Whereas the N-terminal head region consists of a three-helix bundle (\(\alpha\)-helices H3, H7 and H8) with an additional helix H2 added onto one side of this bundle, the
which either contain top-scoring hits belong to a considerable variety of domain folds of IE1 is so far unique. Assigned to any known topology and suggests that the overall fold respectively (Table S2). This indicates that IE1 cannot readily be bundles that partly resemble the IE1 head or stalk region. The eleven monomer (residues 41–393) illustrating the elongated, femur-like fold of IE1. The eleven family members, namely TRIM69 [45] and TRIM5α [46]. These observations suggest a common architecture of TRIM coiled-coils and provide evidence for structural similarities between IE1 and TRIM proteins.

Oligomerization and conservation of primate cytomegalovirus IE1 proteins

RhIE1CORE forms a dimer with C2 point group symmetry in the crystal (Figure 4A), and oligomerization of rhIE1 and hIE1 was confirmed both by gel filtration experiments and co-immunoprecipitation analyses (hIE1) (Figure 4B and C). RhIE1CORE dimerizes with both stalk regions juxtaposed in an antiparallel fashion (Figure 4A). The main-chain conformation differs in the two monomers (Cα-RMSD for all helical segments = 2.13 Å, Figure S5). This deviation originates from a pronounced kink that is observed in one of the two monomers and that causes a repositioning of the loop that interconnects helices H8 to H9 with a concomitant displacement of the N-terminal half of helix H9 (Figure S6). This kink solely occurs in the tetragonal crystal form whereas in the monoclinic space group all four monomers in the asymmetric unit are highly similar. Since this space group transition is triggered by the experimental dehydration of the crystals, we propose that this in situ molecular shape reflects an inherent flexibility of the IE1CORE fold that allows for small readjustments in the packing of the helices.

Intermolecular contacts are formed along the entire rhIE1 length resulting in an extraordinary large interface area (O 3173 Å² per molecule). In the stalk region, these contacts are predominantly hydrophilic, whereas several sparse hydrophobic patches are formed between head regions. Because of its predominantly hydrophilic nature, the dimer interface does not resemble interfaces typically observed in permanent oligomers, suggesting that the dimer could become disrupted upon interaction with binding partners.
This idea is further corroborated by the analysis of IE1\_CORE surface conservation indicating that the dimer interface is not higher conserved than the solvent exposed regions (Figure 4D). Evolutionary conserved surface patches are distributed almost over the entire surface of IE1\_CORE (Figure 4D, blue), whereas non-conserved patches are mainly restricted to loop regions (Figure 4D, red). This indicates that the overall biophysical properties are conserved within the IE1 family of proteins despite the rather low degree of sequence identity. This is in line with the results of a molecular model generated for hIE1 based on the rhIE1 crystal structure (Figure 5A). This model exhibits a good global and local quality further indicating that hIE1\_CORE and rhIE1\_CORE adopt highly similar folds (Figure S7). Due to the observed structural conservation we asked whether rhIE1 is likewise capable of disrupting human PML-NBs. Infection of primary human fibroblasts (HFFs) were transfected with eukaryotic expression plasmids encoding full-length hIE1 or truncated hIE1\_CORE proteins. Surprisingly, while full-length hIE1 exhibited a dispersed nuclear localization and induced a loss of PML-aggregates, all truncated proteins showed a punctate staining pattern colocalizing with PML foci. Based on these data, we conclude that a region within the C-terminal hIE1 IDR is necessary for PML-dispersal. In contrast, hIE1\_CORE alone was sufficient to induce deSUMOylation of PML and the isolated rhIE1 expression was sufficient to redistribute PML (Figure 5D, lower panel) indicating that hIE1 and rhIE1 do not only share biophysical properties but also functional activities.

IE1\_CORE induces deSUMOylation of PML but fails to disperse PML accumulations

In order to study whether the function of hIE1\_CORE differs from that of full-length hIE1, we investigated the subcellular localization of the hIE1\_CORE (Figure 6A). For this purpose, primary human fibroblast cells (HFFs) were transfected with eukaryotic expression plasmids encoding full-length hIE1 or truncated hIE1\_CORE proteins. Surprisingly, while full-length hIE1 exhibited a dispersed nuclear localization and induced a loss of PML-aggregates, all truncated proteins showed a punctate staining pattern colocalizing with PML foci. Based on these data, we conclude that a region within the C-terminal hIE1\_IDR is necessary for PML-dispersal. In contrast, hIE1\_CORE alone was sufficient to induce deSUMOylation of PML in transient expression experiments using 293T cells (Figure 6B). Consistent results were obtained with a whole cell population of HFF cells stably expressing the hIE1\_CORE variant 1–

Figure 3. Structural comparison of the rhIE1 with TRIM25. (A) Overlay of dimeric TRIM25 (in blue and red) with monomeric rhIE1 (gold). (B) Dimeric TRIM25. (C) Topological arrangement of helices H1 to H3 of rhIE1. (D) Structure of the TRIM25 monomer. doi:10.1371/journal.ppat.1004512.g003
Figure 4. Oligomerization of IE1. (A) Dimeric assembly of rhIE1 in the crystal. The two monomers (in cyan and gold) are related by a local 2-fold rotation axis indicated by a black ellipse. (B) Self-association of recombinant IE1 and IE1\textsubscript{CORE} in gel filtration experiments. Overlay of the elution profiles of full length hiIE1(1–491) (blue) and hiIE1\textsubscript{CORE}(14–382) (gold). Full length hiIE1 and hiIE1\textsubscript{CORE} eluted as symmetric main peaks with apparent molecular weights of ∼390 kDa and ∼194 kDa, respectively. It currently remains unclear whether the elution behavior of hiIE1\textsubscript{CORE} merely reflects the elongated molecular shape of the dimer as observed in the crystal structure of rhIE1\textsubscript{CORE} or whether hiIE1\textsubscript{CORE} assembles in solution into oligomers that comprise more than two molecules. Molecular weight estimates are based on commercially available calibration proteins, as indicated by the grey curve. (C) Self-interaction of the IE1\textsubscript{CORE} in human cells. HEK293T cells were co-transfected with expression plasmids encoding Myc-tagged hiIE1(1–382) and FLAG-tagged hiIE1 variants comprising residues 1–382 or 1–377. After cell lysis, immunoprecipitation was performed with an anti-FLAG antibody. Proteins within the cell lysate (input) and co-precipitated PML (IP) were analyzed by Western blotting as indicated. (D) Evolutionary sequence conservation of IE1 mapped onto the surface of rhIE1. The chains of dimeric rhIE1 are shown in space-filled and ribbon representation, respectively. Residues are colored according to their degree of conservation ranging from highly conserved (dark blue) to highly variable (red). The two views differ by a rotation of 180° around a vertical axis.

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Figure 5. Structural and functional similarity between hIE1 and rhIE1. (A) Homology model of hIE1 (blue) superimposed onto the rhIE1 template crystal structure (red). The insertions (green) and deletions (yellow) of hIE1 compared to rhIE1 are mainly located in loop regions and do therefore not disrupt the secondary structure elements. Structure validation using ProSA indicates that the model exhibits a good global and local quality and the respective structural parameters are close to that of the template crystal structure (Figure S7) suggesting that hIE1 and rhIE1 adopt a highly similar fold. (B) Dispersal of PML-NBs after RhCMV infection of primary human fibroblasts (HFFs). HFF cells were infected with RhCMV at an MOI of 0.1 and harvested at indicated times for immunofluorescence analysis of rhIE1 and endogenous PML. Cell nuclei were counterstained with DAPI.
IE1CORE efficiently binds to PML via the coiled-coil domain

Due to the accumulation of hIE1CORE at PML foci, it was attractive to speculate that the two proteins might strongly interact with each other, which was investigated by co-immunoprecipitation (Figure 7A). Intriguingly, while only a trace amount of PML was associated with full-length hIE1, PML was efficiently coprecipitated with hIE1CORE variants. An increased affinity of hIE1CORE for PML was also confirmed by yeast two-hybrid experiments (Figure 7B), which is in line with previous results by Lee et al. (2004) that show an enhanced interaction of PML with an IE1 variant lacking the acidic C-terminus (IE1 1–120) [55]. Having observed a structural similarity between IE1 and coiled-coil domains of TRIM proteins, we asked whether this domain of PML is required for binding of IE1. In a yeast two-hybrid analysis utilizing a series of C-terminal PML deletion mutants we observed that a truncation of the coiled-coil domain abrogated the interaction with IE1 (Figure 7C). To further confirm this finding, coimmunoprecipitation analyses were performed with additional N- and/or C-terminal PML deletions. Importantly, this experiment revealed that the coiled-coil domain of PML was sufficient to mediate an interaction with IE1 (Figure 7D, lower panel, lane 4). Furthermore, we observed that IE1 was also able to bind to TRIM5α suggesting that IE1 targets additional TRIM factors via coiled-coil interactions (Figure 7E).

IE1CORE antagonizes PML-mediated intrinsic immunity during viral infection

Having shown that hIE1CORE binds with high affinity to and deSUMOylates PML, but fails to disrupt PML accumulations, it was important to investigate whether this is sufficient to antagonize PML-NB mediated repression of viral infection. We constructed a recombinant HCMV expressing hIE1 lacking the C-terminal IE1IDR (Figure 8A) and could observe that this virus exhibited a severe defect to disperse PML after infection of HFFs (Figure 8B). Consistent with our results obtained after isolated expression of hIE1CORE, deSUMOylation of both PML and Sp100 was fully preserved (Figure 8C). Most importantly, however, the hIE1CORE-expressing virus replicated nearly as efficiently as wild-type virus expressed in a system where only a trace amount of hIE1 was coprecipitated with hIE1CORE variants. An increased affinity of IE1CORE due to interaction with IE1 (Figure 7C). To further confirm this finding, coimmunoprecipitation analyses were performed with additional N- and/or C-terminal PML deletions. Importantly, this experiment revealed that the coiled-coil domain of PML was sufficient to mediate an interaction with IE1 (Figure 7D, lower panel, lane 4). Furthermore, we observed that IE1 was also able to bind to TRIM5α suggesting that IE1 targets additional TRIM factors via coiled-coil interactions (Figure 7E).

IE1CORE resembles the action of the SUMO-specific protease SENP-1 which also abrogates the SUMOylation of PML, but leaves most PML aggregated [52]. It was previously speculated that IE1 could harbor an intrinsic SUMO protease activity itself or recruit SUMO-specific proteases to the NBs [53]. However, our structural analysis of IE1CORE provides no evidence for the presence of a potential active site with hydrolase activity. Furthermore, in earlier studies no interaction of IE1 with SENPs could be detected, but it was reported that full-length IE1 could still disassemble foci formed by a PML protein with all acidic domain which binds STAT2 thus antagonizing the interferon response [28,29]. This was also confirmed in a complementation experiment after infection of either hIE1- or hIE1CORE-expressing HFFs with an hIE1-deleted HCMV, finally demonstrating that hIE1CORE can efficiently substitute for full-length IE1 during lytic HCMV infection (Figure 8E).

Discussion

The immediate-early protein IE1 of human cytomegalovirus that directly binds to PML is known as an important herpesviral antagonist of PML-NB-mediated intrinsic immunity [15,22,33,36]. However, the structural basis for its function has remained elusive due to the paucity of high-resolution structural information. Here, we present the first crystal structure for the evolutionary conserved primate cytomegalovirus IE1 proteins and demonstrate that a structurally conserved IE1CORE domain is sufficient to antagonize PML-mediated intrinsic immunity. The structure of IE1CORE consists of a femur-shaped bundle of helices, which surprisingly does not share any overall fold similarity with known protein structures. IE1CORE binds with high affinity to PML and efficiently abrogates PML SUMOylation, but fails to disrupt PML accumulations itself. Only upon inclusion of the C-terminal, intrinsically disordered region (IE1IDR) PML dispersal is observed. Thus, our study demonstrates that PML deSUMOylation can be discriminated from PML dispersal. Whereas the first activity is achieved by a distinctly folded IE1CORE domain, the second activity requires inclusion of C-terminal sequences of the IE1IDR region that is highly susceptible to proteolytic degradation and for which we did not observe any stable secondary structure formation. As it has been observed for many intrinsically disordered proteins, folding of the natively disordered IE1IDR region may occur upon binding to a specific interaction partner. First evidence for this comes from a recent study predicting that the chromatin-tethering domain (CTD) at the extreme C-terminus of IE1 forms a β-hairpin when bound to histone proteins [47]. Importantly, IE1CORE is able to release other NB-components like Sp100, hDaxx and ATRX into the nucleoplasm and this correlates with antagonization of NB-mediated repression. This shows that PML dispersal as observed during infection with herpes simplex virus type I and HCMV is not a prerequisite to antagonize the repressive effects of this cellular multiprotein complex on viral gene expression [24,48]. As also suggested by recent findings on the γ-herpesviruses Herpesvirus saimiri and Kaposi sarcoma herpesvirus as well as on the polyomavirus BKV more subtle modifications like the release or degradation of individual NB-components appear to be sufficient [49–51].
SUMOylation sites mutated [53]. Based on this observation, the authors raised the idea that SUMO-independent interference with PML oligomerization followed by exposure of SUMOylated PML to cellular SUMO proteases may account for NB disruption. However, the results of our study argue against such a scenario, since abrogation of PML SUMOylation by IE1CORE was detected while PML aggregates were still present. Thus, IE1 affects its targets via direct, SUMO-independent substrate interaction and this suggests that IE1 does not directly or indirectly act as a hydrolase that specifically targets SUMOylated PML.

Importantly, our study revealed structural similarities between IE1CORE and the crystal structure of the tripartite motif coiled-coil that appears to act as a critical scaffold organizing the biochemical activities of TRIM proteins [44,45]. Moreover, we were able to confirm that the coiled-coil of PML is sufficient for strong binding to IE1. Increasing evidence suggests that TRIM proteins function as E3-ubiquitin ligases in agreement with the family-wide presence of several conserved domains, namely a RING domain followed by two B-boxes and a coiled-coil region [5]. Based on the recently solved crystal structure of the TRIM25 coiled-coil it was shown that TRIM proteins dimerize by forming interdigitating antiparallel helical hairpins that position the N-terminal catalytic RING domain at opposite ends of the dimer and the C-terminal substrate-binding domains at the center [44]. For some of the TRIM members, and among these PML, E3-SUMO instead of E3-ubiquitin ligase activity has been reported [5]. Thus, we would like to propose that IE1CORE, via its strong interaction with the PML coiled-coil, may inhibit an E3-SUMO ligase activity of PML that is required for auto-SUMOylation. Alternatively, IE1 binding to the coiled-coil might block the accessibility of PML for other components of the cellular SUMOylation machinery. Thus, the results of our study favor a model whereby IE1 primarily affects the on-rate of SUMO modification which is also supported by the slow kinetics of IE1-mediated loss of PML SUMOylation [54]. This is different from the ICP0 protein of herpes simplex virus type 1 which induces the rapid degradation of SUMO-conjugated proteins by acting as a SUMO-targeted ubiquitin ligase (STUbL) [21]. Similar to IE1, the adenoviral E4-ORF3 protein which has been shown to form a multivalent matrix via extensive self-interactions, appears to inactivate PML via tight binding [55]. This specific assembly of E4-ORF3 creates avidity-driven interactions that capture PML as well as other tumor suppressors thus disrupting PML bodies. However, in contrast to IE1, the recently solved crystal structure of E4-ORF3 revealed the molecular mechanism of multimerization, but not the exact mode of PML recognition [55].

In this context it should be noted that the nonstructural NS1 protein of influenza A virus has also been shown to target a TRIM protein, TRIM25, via interaction with the coiled-coil domain to inhibit its E3 ligase function [56]. Since TRIM25 catalyzes a critical ubiquitination of the viral RNA sensor RIG-I this constitutes a mechanism by which influenza virus inhibits the host IFN response. Interestingly, we detected that IE1CORE not only binds to PML but also to TRIM5α and a recent publication reported an interaction with TRIM53 [57]. Thus, the unique structure of IE1core may have developed during evolution to facilitate the formation of heteromeric assemblies. The formation of extended coiled-coil interactions would also readily offer an explanation for the finding that single mutations within the conserved SUMOylation patches of IE1 only moderately affect its interaction properties with PML. In contrast, mutations affecting the overall tertiary structure (e.g. IE1 L174P) abrogate the functionality.

Furthermore, it agrees with our observation that rhIE1 can substitute for hIE1 during infection of human cells despite low overall sequence identity. Thus, the size and unique elongated fold of the IE1CORE could have developed during evolution to accommodate efficient binding of PML and possibly other TRIM factors via an extended surface involving coiled-coil interactions. This feature might render the interaction less amenable to mutational escape.

Materials and Methods

Recombinant protein production and purification

All variants of h-, c-, and rhIE1 were recombinantly produced in E. coli strain BL21(DE3) (Novagen) as GST-tagged fusion proteins for in vitro experiments and crystallization. LB media (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were inoculated with freshly transformed E. coli colonies, and cell cultures grown at either 30° or 37°C. Seleno-methionine labeling of rhIE1 (residues 36–395) was achieved by incubation of the cells with non-inducing PAG medium (pre-culture) and auto-inducing PASM-5052 medium (main culture). Cell pellets were resuspended in phosphate buffer and lysed by sonication. Protein purification was achieved by the following steps: a first affinity chromatography (Glutathione sepharose, GE Healthcare, Freiburg), proteolytic cleavage with PreScission protease, a second affinity chromatography and a final size exclusion chromatography (Superdex 200 prepgrade, GE Healthcare). The gel filtration column was pre-equilibrated in 25 mM Tris, 150 mM NaCl, 10 mM DTT, pH 7.5. The samples were separated with an isocratic gradient of 12 column volumes (CV) of the same buffer at a flow rate of 1.5 mL/min. The column was calibrated utilizing the elution peaks of thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa) of...
the Bio-Rad gel filtration standard (Bio-Rad Laboratories, Munich, Germany). The molecular weight of the samples was determined by linear regression. The Kav coefficients of the standard proteins were plotted vs the logarithm of their molecular weights to obtain the calibration curve, with Kav = (Ve-V0)/(Vc-V0), where V0 is the column void volume, Ve is the elution volume and Vc is the geometric column volume. All purification steps were performed in the presence of 10 mM DTT. For the crystallization of variant rhIE1(36–395) the protein was chemically modified by lysine methylation prior to the final size exclusion chromatography step. A 1 mg/mL IE1 protein solution was incubated on ice with 20 μL of 1 M dimethylamine borane.

Figure 7. Interaction of IE1CORE with PML and TRIM5α. (A) Enhanced binding of IE1CORE to PML in coimmunoprecipitation analysis. HEK293T cells were cotransfected with expression plasmids encoding FLAG-hIE1 variants (as indicated) and Myc-PML (isoform VI). Upper two panels: Western blot detection of PML and hIE1 after immunoprecipitation with an anti-FLAG antibody. Lower two panels: detection of PML and hIE1 in cell lysates before precipitation (input). (B) Enhanced binding of IE1CORE to PML in yeast two-hybrid assays. The graph shows the quantification of β-galactosidase reporter activity after coexpression of PML and full-length hIE1 or the hIE1CORE(14–382) in yeast cells. (C) The PML coiled-coil is required for IE1 binding in yeast two-hybrid assays. Full-length hIE1 and hIE1CORE(14–382) were tested for interaction with various C-terminal PML deletion mutants (shown in the upper scheme) by XGal filter lift assays. (D) The PML coiled-coil is sufficient for binding to hIE1CORE in coimmunoprecipitation analysis. HEK293T cells were transfected with various FLAG-tagged PML deletion mutants, as shown in the upper scheme, together with Myc-hIE1CORE(1–382). Upper panel: Western blot detection of hIE1CORE after immunoprecipitation with an anti-FLAG antibody. Lower two panels: Western blot detection of PML variants and hIE1CORE in cell lysates before precipitation (input). (E) Interaction of hIE1CORE with TRIM5α. HEK293T cells were cotransfected with expression plasmids encoding Myc-tagged hIE1CORE(1–382) and either FLAG-tagged PML or rhTRIM5α. Upper panel: Western blot detection of hIE1CORE after coimmunoprecipitation using an anti-FLAG antibody. Lower two panels: Western blot detection of PML, rhTRIM5α and hIE1CORE in cell lysates before precipitation (input). R, RING domain; B, B-boxes; CC, coiled-coil domain; NLS, nuclear localization signal; S, SUMO.

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(DMAB) and 40 μL of 1 M formalin per mL of IE1 solution. After two hours the addition of DMAB and formalin was repeated and, following an additional two-hour incubation, 10 μL of 1 M DMAB per mL of IE1 solution were added, and the solution was incubated at 4°C overnight. The reaction was quenched by adding 125 μL of 1 M Tris/HCl, pH 7.5 per mL of IE1 solution, and the protein was stabilized by addition of 10 mM DTT [58]. Following the final chromatography step, the protein samples were concentrated to 20 mg/ml and stored at 2°C to 20°C in 25 mM Tris/HCl, 1.5 mM NaCl, 15 mM DTT, 1 mM EDTA, pH 7.4 before further usage.

**Limited proteolysis**

Limited proteolysis was performed in order to probe the conformational architecture of the protein [59]. The assay was conducted at 21°C with protein concentrations between 0.2 and 0.5 mg/mL and 0.014 mU subtilisin (Sigma-Aldrich) per μg IE1 protein. Aliquots of 10 μL were taken at different time points, for
example at 1 min, 10 min, 30 min, 60 min, 120 min, 180 min, 240 min and 300 min, mixed with 3.3 µL 4× SDS loading buffer and boiled at 95°C for 5 min to stop the cleavage reaction.

Circular dichroism spectroscopy

Circular dichroism spectra were recorded between 185 and 260 nm from protein samples containing 1.3 µM or 2 µM protein for full-length or truncated IE1, respectively. The measurements were performed in 10 mM potassium phosphate buffer, pH 7.5 with a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) at 20°C with standard sensitivity. The cuvette had a path length of 0.1 cm, the band width was 1.0 nm, the scan speed 20 nm/sec⁻¹, data integration time 1 sec and the data pitch 0.1 nm. All measurements were accumulated ten times and corrected for the sample buffer. Conversion of the data to concentration-independent mean residual weight (MRW) ellipticities [θ]MRW was done as described previously [40].

Crystallization of rhIE1(36–395)

Initial crystallization conditions were identified with a sparse matrix screening approach (Index Screen, Hampton Research, Aliso Viejo, USA) and a Phoenix protein crystallization robot (Art Robbins Instruments, Sunnyvale, USA) [60]. The crystallization conditions were optimized in a hanging drop vapor diffusion setup and involved microseeding. Crystals of diffraction quality were obtained by mixing 1 µL of protein solution with 1 µL of reservoir solution and equilibrating the droplet of 2 µL against 700 µL reservoir solution [0.4 M magnesium formate, 15% (w/v) PEG 3350]. The crystals were soaked in cryo-solution [0.4 M magnesium formate, 15% (w/v) PEG 3350, 15% (v/v) ethylene glycol or 20% (v/v) dimethyl sulfoxide (DMSO)] prior to flash-cooling in liquid nitrogen.

Crystallographic data collection and crystal dehydration

The crystal structure of rhIE1(36–395) was initially solved in space group P2₁ using the following diffraction datasets collected at 100 K at beamline BL14.1 at BESSY II synchrotron (Helmholtz Zentrum Berlin): a native dataset 1 extending to 2.85 Å resolution, a MAD dataset (peak, inflection point, remote) recorded from a gold-soaked crystal diffracting to 3.5 Å and a peak dataset from a seleno-methionine derivatized protein crystal diffracting to 3.1 Å resolution (Table S1). The gold-soaked crystal was prepared by incubating crystals for 3 days in cryo-solution containing DMSO and 2.5 mM KAu(CN)₂. Before flash cooling, the crystals were back-soaked in cryo-solution for several minutes to remove unspecifically bound heavy atoms. All monoclinic datasets are highly isomorphous. The Matthews coefficient is 2.96 (58.42% solvent) when assuming the presence of four rhIE1(36–395) molecules in the asymmetric unit [61]. The final refinement of rhIE1(36–395) was performed against a dataset with space group symmetry P43 extending to 2.3 Å (Table S1). The increase in resolution and concomitant space group change were obtained upon controlled dehydration of the monoclinic crystals from above with the HC1c crystal humidifier device at beamline BL14.3 at BESSY II (Helmholtz Zentrum Berlin). Crystals were first equilibrated against 98% relative humidity before decreasing the humidity in steps of 4% and 10 min equilibration time to a final value of 86% humidity. Upon observation of an increase in diffraction power, the crystals were flash cooled and transferred to beamline BL14.1 for the recording of a complete dataset extending to 2.3 Å resolution (Table S1). All diffraction datasets were processed with XDS and scaled with XSCALE [62].

Structure determination and refinement

Initial protein phases were derived for the monoclinic crystal form using the MAD dataset collected from a gold-soaked crystal (Table S1). The positions of the gold atoms could be readily located with program SHELXD [63]. The non-crystallographic symmetry (NCS) relationship between the 4 monomers, i.e. the presence of two IE1 dimers with C2 point group symmetry, became apparent upon visualization of the gold positions in program COOT and the inspection of the initial electron density maps calculated with program SHELXE [64,65]. The NCS relationship was corroborated by the self-rotation function, calculated with program POLARREF from the CCP4 program suite [66]. The quality of these initial electron density maps could be significantly improved upon phase calculation with program SHARP/autosharp [67,68] and density averaging with program DM [69]. The improved phases also allowed for the identification of the selenium positions in the peak dataset of the seleno-methionine-labeled protein crystal and the inclusion of this dataset in the calculation of the experimental protein phases. An initial atomic model covering a single monomer was then manually built starting from protein fragments derived with program autoSHARP and following the lead of electron density maps calculated with either program autoSHARP or MLPHARE/DM [66,68]. The registration of the protein sequence was obtained from the shape of the local electron density and the positions of the selenium atoms as visualized by an anomalous difference map. These considerations also showed that one gold cation is bound into a free cysteine side chain in each IE1 monomer chain. The model was then stepwise completed, extended to four molecules in the asymmetric unit and refined with program PHENIX [70]. Convergence of the refinement at 2.3 Å in space group P2₁ was facilitated upon inclusion of NCS weights and secondary structure restraints in program PHENIX [70]. The final model of rhIE1(36–395) was obtained after transferring the monoclinic model into the tetragonal unit cell with program PHASER and upon refinement against the 2.3 Å dataset in space group P4₃ (Table S1) [71]. Refinement converged at crystallographic R-factors of 19.73% (Rwork) and 24.96% (Rfree). The space group change that took place upon dehydration can be easily explained by small readjustments in the packing of the IE1 dimers in the crystals.

PDB accession numbers

Coordinates and structure factors for the IE1CORE structure have been deposited in the Protein Data Bank under accession code 4WID.

Bioinformatic analyses

The size of the dimerization interface between the two rhIE1CORE monomers was calculated with the program PISA [72]. The reported value is the average of the buried surface of both chains. The rhIE1CORE monomers shown in Figure S5 were superposed with the program LSQKAB [66]. Only the α-helical segments of the protein as defined in Figure S1 were superimposed. Comparative modeling of hIE1 was performed with MODELLER 9.9 [73] and the resulting model was validated using ProSA [74,75]. Searches for structurally similar proteins were performed with PDBeFold [76]. Since standard parameters did not result in any hits, the following search options were set. (i) The threshold of 70% for the lowest acceptable match in query and target was reduced to 30% and 60%, respectively. (ii) The search was extended to proteins with a different connectivity of their secondary structure elements. Searches were performed independently for chain A and chain B of rhIE1, and the list of hits
was merged. For reasons of clarity, duplicate hits and hits related closely in sequence (>90% identity) were removed from the list. The normRMSD was calculated according to the following equation [77]: \[ \text{normRMSD} = \frac{\text{RMSD} \times \text{max}(\text{L}_1, \text{L}_2) + N_{\text{ali}}}{\text{N}_{\text{tm}}} \]

where RMSD is the root mean square deviation of the superposition of query and target, \( \text{max}(\text{L}_1, \text{L}_2) \) is the number of amino acids of the largest chain in the superposition, and \( N_{\text{ali}} \) is defined by the number of structurally equivalent residue pairs. Sequence conservation was calculated based on a Blosum30 matrix using the MultiSeq Plugin [78] of VMD [79]. The IE1 sequences from the following viruses served as input: human CMV (strain AD169), Rhesus-CMV, Baboon-CMV, Simian-CMV, and Panine-HV2/Chimpanzee CMV (Uniprot-accession-numbers P13202, Q2FAE9, D0UZ7W, Q96682, Q8QRY6). Prediction of intrinsically disordered regions in hIE1, cIE1 and rhIE1 was performed with IUPred [39] using the prediction type “short disorder”. The disorder tendencies in the three IE1 homologs were plotted in one diagram using the rhIE1 amino acid sequence as a reference. The disorder tendencies of the three IE1 homologs were compared to calculate the sequence conservation based on a Blosum30 matrix.

**Crystal Structure of Cytomegalovirus IE1 Protein**

The AD169-based HCMV bacterial artificial chromosome (BAC) HB13 was used for recombination-based genetic engineering of AD169/hIE1 1–382 and AD169/AhIE1. AD169/hIE1 1–382 was constructed by introducing a stop codon into the hIE1 gene replacing residue 383. For this purpose, the two-step red-mediated recombination technique was used [83], which uses the kanamycin gene as a first selection marker. The linear recombination fragment was generated by PCR using primers 5′BAC_short and 3′BAC_hIE1_382 (Table S3), and pEPkan-S (kindly provided by K. Osterrieder, Berlin) as template DNA. The PCR product was treated with DpnI, gel purified, and subjected to a second round of PCR amplification using primers 5′BA_C_hIE1_382 and 3′BAC_hIE1_382_short (Table S3). For homologous recombination, the PCR fragment was transformed into Escherichia coli strain GS1703 (a gift of M. Mach, Erlangen) already harboring HB15, and bacteriophage λ red-mediated recombination was conducted as described elsewhere [83]. To identify positive transformants, the bacteria were plated on agar plates containing 30 μg/mL kanamycin (first recombination) or 30 μg/mL chloramphenicol and 1% arabinose (second recombination) and incubated at 32°C for 2 days. BAC DNA was purified from bacterial colonies growing on these plates and was further analyzed by PCR, restriction enzyme digestion and direct sequencing. For construction of the AD169/AhIE1 BAC by homologous recombination, a linear recombination fragment, comprising a kanamycin resistance marker along with 5′ and 3′ genomic sequences, was generated by PCR amplification using pKD13 as template and primers 5′Intron3/pKD13 and 3′Exon4/pkd13 (Table S3). This fragment was used for electroporation of competent Escherichia coli strain DH10B harboring HB15 and recombination was performed as described previously in order to delete exon 4 of the IE1 gene [84]. The integrity of the resulting recombinant BAC was confirmed by PCR, restriction enzyme digestion and direct sequencing.

For reconstitution of recombinant AD169, HFFs were seeded in six-well plates (3×10^5 cells/well) were co-transfected with 1 μg of purified BAC DNA, 0.5 μg of the pp71 expression plasmid pcB6-pp71, and 0.5 μg of a vector encoding the Cre recombinase using FuGENE6 transfection reagent (Promega, Mannheim, Germany). Transfected HFFs were propagated until viral plaques appeared, and the supernatants from these cultures were used for further virus propagation.

**Lentivirus transduction and selection of stably transduced cells**

For the generation of HFF cells stably expressing full length hIE1 or hIE1 1–382, replication-deficient lentiviruses were generated using pLKO-based expression vectors. For this purpose, HEK293T cells seeded in 10 cm dishes (5×10^6 cells/dish) were co-transfected with a pLKO vector encoding either full length hIE1 or hIE1 1–382 together with packaging plasmids pLP1, pLP2, and pLP/VSV-G using the Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany). Viral supernatants were harvested 48 h after transfection, cleared by centrifugation, filtered, and stored at −80°C. Primary HFFs or telomerase-immortalized HFFs were incubated for 24 h with lentivirus supernatants in the presence of 7.5 μg/mL polybrene (Sigma-Aldrich, Deisenhofen, Germany).
Stably transduced cell populations were selected by adding 500 µg/mL geneticin to the cell culture medium.

**Transfection**

HFF cells were transfected with the DNA transfection reagent FuGENE6 (Promega, Mannheim, Germany). One day before transfection, 3×10^5 cells were seeded into six-well dishes. DNA content and transfection procedure were according to the instructions of the manufacturer. 48 hours after transfection, cells were harvested for further analyses. HEK293T cells were transfected by applying the standard calcium phosphate precipitation method. For this, 5×10^5 to 5×10^6 HEK293T cells were seeded into six-well dishes or 10 cm dishes one day before transfection. For Western blot analyses and coimmunoprecipitations, 1 to 10 µg of plasmid DNA were used for each transfection reaction. At about 16 hours later, the cells were washed two times with PBSO and provided with fresh medium. 48 hours after transfection, cells were harvested for further analyses.

**Antibodies**

Monoclonal antibodies used for immunofluorescence and Western blot analyses were: α-IE1 CH413 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-UL112/113 M23, α-UL44 BS510 (kindly provided by B. Plachter, Mainz, Germany), α-UL69 69–66, α-FLAG M2 (Sigma-Aldrich, Deisenhofen, Germany), α-Myc 9E10, α-β-actin AC-15 (Sigma-Aldrich), α-PML PG-M3 (Santa Cruz). Polyclonal antibodies used for immunofluorescence and Western blot analyses were: α-rheus IE1 (a kind gift from M. Mach, Erlangen, Germany), α-PML #4 (a kind gift from P. Hemmerich, Jena, Germany), α-PML H238 (Santa Cruz), α-PML A301–167A (Bethyl Laboratories, Montgomery, TX, USA), α-PML A301–168A (Bethyl Laboratories), α-Sp100 #2 (a kind gift from P. Hemmerich, Jena, Germany), α-Sp100 GH3 (kindly provided by H. Will, Hamburg, Germany), α-hDaxx C-20 (Santa Cruz), α-ATRX H-300 (Santa Cruz). Secondary antibodies used for immunofluorescence and Western blot analyses were; Alexa Fluor 488-/555-/647-conjugated secondary antibodies for indirect immunofluorescence experiments were purchased from Molecular Probes (Karlsruhe, Germany), horseradish peroxidase-conjugated anti-mouse/-rabbit secondary antibodies for Western blot analyses were obtained from Dianova (Hamburg, Germany).

**Indirect immunofluorescence**

HFF cells grown on coverslips in six-well dishes (3×10^5 cells/well) were washed twice with PBSO at 48 hours after transfection or at various times after virus infection. Cells were fixed with a 4% paraformaldehyde solution for 10 min at room temperature (RT) and then washed for two times. Permeabilization of the cells was achieved by incubation with 0.2% Triton X-100 in PBSO on ice for 20 min. Cells were washed again with PBSO over a period of 5 min and incubated with the appropriate primary antibody diluted in PBSO-1% FCS for 30 min at 37°C. Excessive antibodies were removed by washing four times with PBSO, followed by incubation with the corresponding fluorescence-coupled secondary antibody diluted in PBSO-1% FCS for 30 min at 37°C. The cells were mounted using the DAPI-containing Vectashield mounting medium (Alexis, Grünberg, Germany) and analyzed using a Leica TCS SP5 confocal microscope, with 488 nm, 543 nm, and 633 nm laser lines, scanning each channel separately under image capture conditions that eliminated channel overlap. The images were exported, processed with Adobe Photoshop CS5 and assembled using CorelDraw ×3. In order to quantify PML-NB disruption in infected HFFs, 150 cells were analyzed for the presence of PML dots.

**Immunoblotting**

Lysates from transfected or infected cells were prepared in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, separated on sodium dodecyl sulfate-containing 8 to 15% polyacrylamide gels, and transferred to nitrocellulose membranes. Chemiluminescence was detected according to the manufacturer’s protocol (ECL Western blot detection kit; Amersham Pharmacia Biotech).

**Coimmunoprecipitation**

Transfected HEK293T cells (1×10^5 or 5×10^5) were lysed for 20 to 40 min at 4°C in 800 µL of CoP buffer (30 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 µg/mL of aprotonin, 2 µg/mL of leupeptin, and 2 µg/mL of pepstatin). After centrifugation, aliquots of each sample were taken as input controls and the remaining supernatant was incubated with anti-FLAG antibody M2 coupled to protein-A-sepharose beads for 2 h at 4°C. The sepharose beads were collected by centrifugation and washed five times in 1 mL CoP buffer. Finally, the immunoprecipitated proteins were recovered by boiling in 4× SDS sample buffer and protein complexes were analyzed by SDS-PAGE and Western blotting.

**Yeast two-hybrid analysis**

*Saccharomyces cerevisiae* Y153 was used in a two-hybrid system. Both the plasmid pGBT9 (Clontech, Mountain View, CA) encoding the GAL4-DB (Trp+) fusion and the plasmid pGAD424 (Clontech, Mountain View, CA) encoding the GAL4-A fusion (Leu+) were introduced into Y153 cells using a modified lithium acetate (LiAc) method. For this, cells were grown overnight in YAPD medium, pelleted and treated with LP-mix (40% w/v PEG 4000, 0.15 M LiAc, 10 mM Tris/HCl pH 7.5, 1 mM EDTA pH 8.0) and DMSO. Single-stranded carrier-DNA as well as both plasmids were added to the yeast cells. This step was followed by incubation at room temperature and subsequent incubation at 42°C. Thereafter, the cells were plated on minimal selection agar lacking Trp and Leu. For rapid *in situ* assays of lacZ expression from yeast colonies, an XGal filter assay was used. Nitrocellulose filters were laid onto the plate and allowed to wet completely, then lifted off the plate and placed in liquid nitrogen to permeabilize the cells. The filters were removed and placed cell side up in a petri dish containing Whatman Paper soaked with Z buffer containing β-Mercaptoethanol and XGal. The filters were incubated at 30°C and constantly analyzed for the development of a positive blue color. For quantitation of the β-galactosidase activity in the yeast cells three colonies were picked and grown in medium also lacking Trp and Leu. The next day, the optical density was measured at 600 nm. After pelleting the culture, the cells were resuspended in Z buffer and permeabilized using chloroform and 0.1% SDS. The β-galactosidase activity within the cells was assayed by the standard method using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The reaction was stopped by adding Na2CO3 and the absorbance was measured at OD405. The unit of β-galactosidase was defined as (1.000 × OD405)/(t × v × OD600) (t, reaction time [min]; v, culture volume [mL]). The β-galactosidase activity for each sample was corrected for background by subtracting the signal of the empty vectors.

**Multistep growth curve analysis**

HFF cells were seeded into six-well dishes at a density of 3×10^4 cells/well and infected the following day with wt AD169 at an MOI of 0.01 and equivalent genome copies of AD169/hIE1 1–382 and AD169hIE1. Triplicate samples of the infected cell
supernatants were harvested at 2, 4, 6, 8 and 11 days after inoculation and subjected to lysis by proteinase K treatment. Thereafter, all samples were analyzed for the amount of genome copy numbers by quantitative real-time PCR (TaqMan-PCR) using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) together with the corresponding software SDS (sequence detection system) [85]. For quantification of the viral DNA load, a sequence region within the gB gene locus was amplified using primers 5’gB_forw and 3’gB_rev along with the fluorescence labeled probe CMV gB FAM/TAMRA also directed against the gB gene region. In parallel, the cellular DNA amount was quantified using primers 5’Alb and 3’ Alb together with a fluorescence labeled probe, Alb FAM/TAMRA, specific for the cellular albumin gene. Real-time PCR was performed in 96-well plates being compatible with the ABI Prism sequence detector. For the determination of reference Ct values (cycle threshold), serial dilutions of the respective standards (10^7–10^1 DNA molecules of gB or albumin) were examined by PCR reactions in parallel. The 20 μL reaction mix contained 5 μL sample or standard DNA solution together with 10 μL 2× TaqMan PCR Mastermix (Applied Biosystems, Foster City, CA, USA), 1.5 μL of each primer (5 μM stock solution), 0.4 μL of probe (10 μM stock solution), and 1.6 μL of H₂O. The thermal cycling conditions consisted of two initial steps of 2 min at 50°C and 10 min at 95°C followed by 40 amplification cycles (15 sec at 95°C, 1 min 60°C). The viral genome copy numbers and albumin copy numbers were subsequently calculated using the sample-specific Ct value when set into relation to the standard serial dilutions.

Complementation assay

For analysis of complementation by immunofluorescence staining, control HFFi cells as well as HFFi cells expressing hIE1 or hIE1 1–382 were infected with AD169AhIE1 at an MOI of 0.01. Triplicate samples of infected cells were fixed at 48 h post infection and subjected to immunostaining of UL44. Images of approximately 500 cells per sample were taken and the number of UL44-positive cells was determined via measuring of mean gray values using the ImageJ software (version 1.47). For analysis of complementation by Western blotting, normal HFFi cells as well as HFFi cells expressing hIE1 or hIE1 1–382 were infected with AD169AhIE1 at an MOI of 0.05 in triplicate, and were harvested 72 h later for detection of UL44, UL69, IE1 and β-actin.

Supporting Information

Figure S1 Sequence alignment of homologous IE1 proteins: hIE1 of HCMV (strain AD169, AC146999), cIE1 of chimpanzee CMV (panine herpesvirus 2, NC003521.1), and rhIE1 of rhesus macaque CMV (ceropithecine herpesvirus 8, DQ120516.1). Sequence conservation is indicated by gray shading (dark gray: highly conserved residues, medium gray: strongly similar residues, light gray: weakly similar residues). Residues as resolved by the crystallographic symmetry averaging were superimposed with an r.m.s. deviation of 2.13 Å. The area with the highest deviation, namely, the loop between helices H8 and H9, is marked by a red box (see also Figure S6). The molecules were superposed with the program LSQKAB [66].

Figure S2 The CD spectra of full-length hIE1(1–491) and hIE1(14–382) are shown in blue and gold, respectively (A). The difference spectrum obtained by subtracting the data of hIE1(14–382) from hIE1(1–491) is shown in green (B). The difference spectrum exhibits strong random coil characteristics with its minimum at a wavelength around 200 nm and therefore confirms the intrinsically disordered nature of the terminal regions. (TIF)

Figure S3 Stereo image of a representative section of the experimentally phased electron density map used for model building. Protein phases in space group P2₁ were derived from a 3.5 Å MAD dataset collected from a gold-soaked crystal and combined with 3.1 Å data from a SeMet SAD dataset collected at the peak wave-length of Se (Table S1). Extensive four-fold non-crystallographic symmetry averaging was applied. The protein density (contoured at 1σ) is shown in blue and the anomalous density calculated from the anomalous difference signal in the SeMet peak dataset (contoured at 3σ) in orange. The helical structure of the protein is clearly visible and the positions of the Se-atoms coincide with the locations of the peaks in the anomalous density map. (TIF)

Figure S4 Multiple sequence alignment of the coiled-coil region of different TRIM proteins. The a and d position of the heptad and hendecad repeats and the h position present only in the hendecad repeats are highlighted.

Figure S5 Stererepresentation showing the superposition of the two monomers that are present in dimeric rhIE1CORE in the final refined structure in space group P4₃. When considering the Cα positions from the α-helical segments, the two monomers can be superimposed with an r.m.s. deviation of 2.13 Å. The area with the highest deviation, namely, the loop between helices H8 and H9, is marked by a red box (see also Figure S6). The maps were generated with program PHENIX and residues Glu276 to Thr293 omitted during refinement and subsequent difference density calculation. The σA-weighted mFo-DFc electron density is displayed at a 3σ level.

Figure S6 Simulated annealed OMIT difference density map illustrating a difference in the kink of the loop formed between helices H8 and H9 in the two monomers (panel A and B) of dimeric rhIE1CORE (see also Figure S5). The maps were generated with program PHENIX and residues Glu276 to Thr292 omitted during refinement and subsequent difference density calculation. (TIF)
Table S1: Data collection, phasing and refinement statistics.

| Crystal Structure of Cytomegalovirus IE1 Protein |

Table S2: List of structurally similar proteins of IE1 sorted by their Z-score in descending order. For each hit the PDB code, the RMSD of the overlay, and the number of structurally equivalent residues ($N_{eq}$) is given. Generally, the number of equivalent residues is rather low covering less than 50% of the total length of IE1. This demonstrates that the overall fold in IE1 is rather unique and there are only local structural similarities to other helical proteins. These local hits were further inspected based on their CATH-classification. Most of the hits from the present search are classified as α-helical orthogonal bundle (code 1.1.0), or up-down bundle (code 1.2.0). Comparison of the third number of the CATH-code shows that these proteins belong to at least 18 different folds indicating that IE1 cannot readily be assigned to any known topology. This is further corroborated by the calculation of a normalized rmsd (normRMSD). For all hits listed in Table S2 the normRMSD values are significantly larger than the threshold of 5 Å [77], which indicates that a protein is significantly structurally different from those proteins previously deposited in the PDB.

Table S3: List of oligonucleotides.

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Author Contributions

Conceived and designed the experiments: TS YAM. Performed the experiments: MSc SK VO EMS RM NR MSe YAM JDS HS. Analyzed the data: SK MSe YAM JDS HS MSc EMS NR TS. Wrote the paper: MSc SK HS YAM TS.

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