Characterization of protein–protein interaction domains within the baculovirus Autographa californica multiple nucleopolyhedrovirus late expression factor LEF-3

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Autographa californica nucleopolyhedrovirus late expression factor 3 (LEF-3) is required for late viral gene expression probably through its numerous functions related to DNA replication, including nuclear localization of the virus helicase P143 and binding to ssDNA. LEF-3 appears to interact with itself as a homo-oligomer, although the details of this oligomeric structure are not yet known. To examine LEF-3–LEF-3 interactions, a bimolecular fluorescent protein complementation assay was used. Pairs of recombinant plasmids expressing full-length LEF-3 fused to one of two complementary fragments (V1 or V2) of a variant of yellow fluorescent protein named ‘Venus’ were constructed. Plasmids expressing fusions with complementary fragments of Venus were co-transfected into Sf21 cells and analysed by fluorescence microscopy. Co-transfected plasmids expressing full-length V1–LEF-3 and V2–LEF-3 showed positive fluorescence, confirming the formation of homo-oligomers. A series of truncated V1/V2–LEF-3 fusions was constructed and used to investigate interactions with one another as well as with full-length LEF-3.

Supplementary methods and a table are available with the online version of this paper.
DNA replication in the absence of LEF-3 (Chen & Carstens, 2005). Through the use of a lef-3 partial knockout bacmid, we demonstrated that only aa 1–125 of LEF-3 was sufficient for interacting with P143 and supporting viral DNA replication and late gene transcription (Yu & Carstens, 2010). Thus, LEF-3 is an important multi-functional protein involved in regulating both virus DNA replication and transcription.

In the current study, we investigated the interaction potential of LEF-3 with itself and identified regions responsible for these interactions. Direct visualization of protein–protein interactions in living cells and organisms has become key to understanding fully their intracellular roles. We exploited a bimolecular fluorescence protein complementation (BiFC) assay, involving the fusion of one of two components of a yellow fluorescent protein called Venus1 (V1, aa 1–158) and Venus2 (V2, aa 159–239) (Nyfeler et al., 2005; Shyu et al., 2006) to various regions of LEF-3 to investigate interacting domains important for oligomerization. A series of plasmids was prepared, expressing full length, truncations or deletions within the AcMNPV lef-3 gene fused at the N terminus with the coding regions of either V1 or V2, separated by a 10 aa linker (GGGGSG)2 necessary to provide flexibility between V1/V2 and the interaction partners (Fig. 1a) (Nyfeler et al., 2005). The V1-linker and V2-linker subunits were amplified from pcDNA 3.1/zeo(+)Venus1[GCN4Zip] and pcDNA 3.1/zeo(+)Venus2[GCN4Zip] (from Dr Stephen Michnick, Université de Montréal, Canada). If two tagged proteins associate in close proximity, the complementary V1 and V2 fragments fold into an active structure detectable by fluorescence microscopy (Remy & Michnick, 2007). The advantage of this system over other approaches such as yeast two-hybrid screens or pull-down assays is that the interactions can be monitored in vivo in cells, with no restriction to any particular cellular compartment where the interaction might take place (Morell et al., 2009). Details of plasmid construction along with a table of primer sequences used to make these constructs are provided as supplementary materials (available in JGV Online).

Recombinant plasmids were tested for expression of expected proteins by transfecting Sf21 cells with plasmid DNA and analysing immunoblots of cell lysates harvested at 24 h post-transfection (p.t.). With the exception of pBSV1Aclef-3(2–83) and pBSV2Aclef-3(2–83), which express a small region not detected with anti-LEF-3 antibody (Chen & Carstens, 2005), all plasmids expressed the expected fusion proteins (Fig. 1b, c). The pBSV1Aclef-3(2–125) construct consistently expressed the product at the highest level of all the fusion proteins from cells transfected with the same amount of DNA, although transfection with pHSEHAclef-3 was always expressed at even higher levels. Interestingly, we have previously demonstrated that the LEF-3 aa 2–125 region supports higher levels of protein synthesis and DNA replication than the LEF-3 aa 1–189 region suggesting that there is something unusual about the LEF-3 aa 2–125 region that leads to enhanced protein expression or stability (Yu & Carstens, 2010).

To determine whether any regions within LEF-3 were able to interact, each plasmid construct was first singly transfected into Sf21 cells to ensure that the Venus fragments did not fluoresce independently. All constructs used in these studies produced only background fluorescence when transfected alone (data not shown). When pBSV1Aclef-3 and pBSV2Aclef-3 expressing full-length LEF-3 were co-transfected, nuclear fluorescence was seen (Fig. 2). These results support the hypothesis that LEF-3 self-associates as a homo-oligomer and indicated that the BiFC assay could be exploited to determine the specific interacting domains within LEF-3. When plasmids expressing V2 fused with either the N-terminal [pBSV2Aclef-3(2–189)] or C-terminal [pBSV2Aclef-3(190–385)] halves of LEF-3 were co-transfected with pBSV1Aclef-3, nuclear fluorescence was also seen, demonstrating that either of these regions could interact with the full-length protein (Fig. 2). When pBSV1Aclef-3(2–189) and pBSV2Aclef-3(2–189) or pBSV1Aclef-3(190–385) and pBSV2Aclef-3(190–385) were co-transfected, again fluorescence was observed (Fig. 2). The fluorescent signal was nuclear when the LEF-3 aa 2–189 region was expressed while the LEF-3 aa 190–385 region, lacking a NLS sequence, produced cytoplasmic fluorescence because the LEF-3 aa 2–189 region carries the LEF-3 NLS sequence (Au et al., 2009). These data indicated that two separate domains of LEF-3, representing roughly half the full-length protein, were able to self-interact, and, in the case of the N-terminal region, also interact with the cellular nuclear transport system. The positive fluorescence seen from co-expression of V1– and V2–LEF-3(aa 190–385) indicated that DNA is not required for oligomerization of LEF-3 since these polypeptides interacted but produced cytoplasmic fluorescence. When pBSV1Aclef-3(2–189) and pBSV2Aclef-3(190–385) were co-transfected, positive nuclear fluorescence was detected (Fig. 2), indicating that the interaction was direct, resulting in transport of LEF-3(aa 190–385) into the nucleus. Two separate regions including aa 39–104 and 183–256 have been predicted to carry ssDNA binding activity, and it was suggested that these separated domains might form the ssDNA binding site (Mikhailov et al., 2006). Our data demonstrating that the two halves of LEF-3 can interact support this suggestion. Whenever the LEF-3 aa 2–189 region was co-expressed with full-length LEF-3, the signal was noticeably punctate and this was seen when either V1– or V2–LEF-3(aa 2–189) was co-expressed with full-length LEF-3. This appearance did not occur when only V1– and V2–LEF-3(aa 2–189) were co-expressed so this may represent some aberrant interaction that results in unusual deposition of the oligomer in the nucleus.

To investigate intra-protein regions that may be involved in interactions within LEF-3, a series of co-transfections was carried out with a plasmid expressing only the N-terminal 83 aa, pBSV1Aclef-3(2–83). To ensure that interactions observed were not an artefact of fusion with one specific Venus domain, reciprocal V1 and V2 constructs expressing full-length LEF-3 were tested against...
Fig. 1. Venus constructs and their expression. (a) A schematic representation of constructs expressed from the plasmids used is shown below a summary of predicted LEF-3 functional regions (see text for references). In addition to full-length V1– and V2–LEF-3, constructs containing partial regions of LEF-3 including LEF-3(aa 2–189), LEF-3(aa 190–385), LEF-3(aa 2–125), and site-specific deletions including LEF-3(D1–56), LEF-3(D14–37) and LEF-3(D126–189) fused to V1 and V2 are shown. Sf21 cells were transfected with various plasmid DNAs and then heat shocked at 42°C for 30 min at 22 h.p.t. Cell lysates, collected at 24 h.p.t. were analysed on immunoblots using polyclonal anti-LEF-3. (b) Plasmids encoding V1– and V2–LEF-3 full-length (lanes 2 and 3), aa 2–189 (lanes 4 and 5), aa 2–125 (lanes 6 and 7) and aa 190–385 (lanes 10 and 11) all expressed proteins of the expected size. No product was detected from plasmids encoding V1– and V2–LEF-3 aa 2–83 (lanes 8 and 9), because the small product lacks an epitope for the anti-LEF-3 antibody (Chen & Carstens, 2005). (c) Plasmids encoding V1–LEF-3 with aa 1–56 deleted (lane 2), and V1– or V2–LEF-3 with aa 14–37 (lanes 3 and 4), aa 57–189 (lanes 5 and 6) or aa 126–189 (lanes 7 and 8) deleted also expressed the expected polypeptides. In (b) and (c), pHSEHAclef-3 was included as a positive control recognized by the anti-LEF-3 antibody (lane 1). M represents mock transfected cell extracts.
plasmids expressing either the V1 or V2 fusions with LEF-3(aa 2–83). Both combinations produced nuclear fluorescent signals (Fig. 3a), clearly showing that this small region was expressed, even though it was not detected by immunoblotting (Fig. 1), and was able to interact with whole LEF-3. We then tested other regions within LEF-3 for their ability to interact with LEF-3(aa 2–83) or C-terminal (aa 190–385) halves of LEF-3 and prepared for fluorescence microscopy at 24 h p.t. Cells were treated with Hoechst stain to reveal nuclei (Hoechst). Green fluorescence indicated an interaction between the two expressed proteins (Venus). Hoechst and Venus images were merged to reveal the intracellular localization of fluorescence (Merge). All combinations revealed nuclear fluorescence except V1– and V2–LEF-3(aa 190–385) (C-terminal half), which showed positive fluorescence localized in the cytoplasm.

The results suggest that the structure of the LEF-3 homooligomer may be quite complex, as also implied by a previous structural analysis (Mikhailov et al., 2006). At physiological temperatures, LEF-3 had an estimated z-helix content above 40%, but this was reduced to about 20% when LEF-3 was incubated at 50 °C. The transition was quite sharp around 50°C, so the 42°C heat shock employed in our experiments probably did not affect the physical structure of LEF-3. However, the interaction of isolated domains of LEF-3, in the absence of full-length LEF-3, are compatible with the heat-induced changes in oligomerization patterns of LEF-3, where large molecular mass complexes were observed after heat treatment (Mikhailov et al., 2006). The ability of the small domain aa 2–83 to interact both with full-length LEF-3 and with other domains within LEF-3 such as aa 2–125, aa 2–189 and aa 190–385 suggests that LEF-3 may consist of several structural domains. It remains to be determined whether these domains are associated with specific independent functions. The BiFC assay also demonstrated that, when the LEF-3 NLS was intact on only one of the interacting partners, it was sufficient for nuclear transport of the whole

and clearly showed that a protein–protein interaction between the N-terminal aa 2–83 and the C-terminal half was strong enough to result in its nuclear localization. The signal obtained with region aa 2–125 was consistently stronger than with aa 2–189, supporting our previous suggestion that aa 126–189 may carry a motif inhibitory to protein–protein interactions (Yu & Carstens, 2010). The differences in the intensity of the observed fluorescence in positive cells could also be related to differences in the protein expression levels of the various constructs as shown in Fig. 1. These results were consistent with different plasmid DNA preparations and at least three BiFC assays.

We also tested the effect of deletions within LEF-3 on the ability of constructs to interact with whole LEF-3 by co-transfecting full-length V2–LEF-3 with constructs expressing proteins where aa 1–56 [pBSV1Aclef-3(D1–56)], aa 14–37 [pBSV1Aclef-3(D14–37)] or aa 126–189 [pBSV1Aclef-3(D126–189)] were deleted. We have shown previously that deletion of aa 14–37 or aa 1–56 removed the NLS, resulting in cytoplasmic localization (Au et al., 2009). Each of these mutant constructs gave nuclear fluorescence when co-expressed with full-length LEF-3 (Fig. 3b), demonstrating that the interacting protein complexes were successfully transported to the nucleus. Deleting aa 126–189 from LEF-3 resulted in punctate fluorescence when co-transfected with full-length LEF-3, again suggesting unusual aggregation of this particular complex. Together, these data demonstrated that full-length LEF-3 interacted directly with all of the truncated and deleted LEF-3 constructs in the cytoplasm and these complexes were rapidly transported to the nucleus. Co-transfection of pBSV1Aclef-3(D14–37) and pBSV2Aclef-3(D14–37) resulted in cytoplasmic fluorescence, supporting the hypothesis that the LEF-3 NLS is located within this region (Au et al., 2009). The NLS is obviously not essential for oligomerization of LEF-3.

Fig. 2. BiFC assays of full-length and the N-terminal and C-terminal halves of LEF-3. Sf21 cells were co-transfected with plasmids expressing V1 or V2 fused with either full-length or the N-terminal (aa 2–189) or C-terminal (aa 190–385) halves of LEF-3 and prepared for fluorescence microscopy at 24 h p.t. Cells were treated with Hoechst stain to reveal nuclei (Hoechst). Green fluorescence indicated an interaction between the two expressed proteins (Venus). Hoechst and Venus images were merged to reveal the intracellular localization of fluorescence (Merge). All combinations revealed nuclear fluorescence except V1– and V2–LEF-3(aa 190–385) (C-terminal half), which showed positive fluorescence localized in the cytoplasm.

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complex. This reveals that LEF-3 (with an intact NLS) is capable of transporting another molecule of LEF-3 (without an intact NLS) to the nucleus, much like whole LEF-3 transports P143 to the nucleus (Chen & Carstens, 2005; Wu & Carstens, 1998). We are now investigating whether LEF-3 may be responsible for nuclear transport of other baculovirus proteins. If so, it will highlight the important role that LEF-3 plays in regulating the virus replication cycle in addition to acting as an SSB protein during replication.

Fig. 3. BIFC assays of LEF-3 deletion constructs. Co-transfected Sf21 cells were prepared as described in Fig. 2. (a) Cells were co-transfected with plasmids expressing V1–LEF-3(aa 2–83) and other small regions of LEF-3 fused with V2 including aa 2–83, 2–189, 190–385 and 2–125. As a control, both full-length V1– and V2–LEF-3 were co-transfected with V2– or V1–LEF-3(aa 2–83), to demonstrate that both of these constructs expressed proteins capable of interaction with full LEF-3. Only V1– and V2–LEF-3(aa 2–83) did not produce any signal when co-expressed, indicating that this small region did not interact with itself. (b) Cells were co-transfected with plasmids expressing full-length V2–LEF-3 and V1 fusions of LEF-3 with deletions of aa 1–56, 14–37 or 126–189, or with plasmids expressing V1– and V2–LEF-3 constructs with aa 14–37 deleted. All combinations with full-length LEF-3 produced positive nuclear fluorescence while the deletion of aa 14–37 (the NLS sequence) on both the V1– and V2–LEF-3 fusions resulted in cytoplasmic fluorescence.

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References


