POSH, a scaffold protein for JNK signaling, binds to ALG-2 and ALIX in Drosophila

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Abstract Plenty of SH3s (POSH) functions as a scaffold protein for the Jun N-terminal kinase (JNK) signal transduction pathway, which leads to cell death in mammalian cultured cells and Drosophila. Here, we show that POSH forms a complex with Apoptosis-linked gene-2 (ALG-2) and ALG-2-interacting protein (ALIX/AIP1) in a calcium-dependent manner. Overexpression of ALG-2 or ALIX in developing imaginal eye discs resulted in roughened or melanized eyes, respectively. These phenotypes were enhanced by co-overexpression of POSH. We found that overexpression of either gene could induce ectopic JNK activation, suggesting that POSH/ALG-2/ALIX may function together in the regulation of the JNK pathway.

Keywords: Plenty of SH3s; Apoptosis-linked gene-2; ALG-2-interacting protein X; c-Jun N-terminal kinase; Cell death

1. Introduction

The Jun N-terminal kinase (JNK) signaling pathway plays an important role in a number of biological processes, including cell growth, differentiation, apoptosis and immune responses. The pathway involves sequential activation of Rac1/Cdc42, MLKs, MKKs and JNKs, eventually resulting in activation of the transcription factor c-Jun [1]. In mammals, plenty of SH3 domains and a RING finger motif are well conserved [7]. In Drosophila, overexpression of POSH during development produces various defects in the adult morphology, such as loss of anterior wing crossveins, notched wings and disordered hair polarity [7]. These phenotypes are suppressed by mutations of basket (bsk, Drosophila JNK) or hemipterous (hep, MKK). Furthermore, overexpression of POSH induces expression of puckered (puc), a target gene of the JNK signaling pathway. A loss-of-function mutation in POSH was shown to be defective in the innate immune system [8]. In this context, POSH interacts with TAK1, an MLK responsible for activation and termination of immunity signaling. Thus, it is clear that POSH mediates the activation of the JNK signaling pathway in Drosophila.

In the present study, we identified and characterized Drosophila apoptotis-linked gene-2 (ALG-2) and ALG-2 interacting protein X (ALIX) as POSH-interacting proteins. ALG-2, encoding a calcium-binding protein that contains five EF-hand motifs, was previously identified as an essential gene for T-cell apoptosis induced by Fas, T cell receptors or glucocorticoid [9]. ALIX, also called AIP1, was isolated through yeast two-hybrid screening by two independent groups [10,11]. ALG-2 and ALIX interact with the src homology 3 (SH3) domain-containing expressed in tumorigenic astrocytes (SETA) gene, which is expressed at high levels in malignant astrocytes in culture and gliomas in the adult brain [12]. We demonstrate that POSH can form a complex with ALG-2 and ALIX both in vitro and in vivo. Overexpression of either ALG-2 or ALIX activates the JNK signaling pathway and leads to morphological defects, which are enhanced by co-expression of POSH. Our results suggest that POSH exerts at least some of its functions through its interactions with ALIX and ALG-2.

2. Materials and methods

2.1. cDNA cloning and fly stocks

cDNAs corresponding to the coding regions of ALG-2 and ALIX were obtained by reverse transcription-polymerase chain reaction (RT-PCR) using mRNAs isolated from adult Drosophila, and subcloned into the EcoRV site of pBluescript KS+ (P-ALG-2 5′: CAAAATGGCCTACCTGAGTAACTGGTAG-3′; P-ALIX 5′: TTACCAGCATCAGGTGGCTTCTGGTTG; P-ALG-2 3′: CTACCTGAGTAACTGGTAG-3′; P-ALIX 3′: CTACCTGAGTAACTGGTAG-3′) as POSH-interacting proteins.

Abbreviations: POSH, plenty of SH3s; ALG-2, apoptosis-linked gene-2; ALIX, ALG-2-interacting protein X; JNK, c-jun N-terminal kinase; RT-PCR, reverse transcription-polymerase chain reaction; UAS, upstream activating sequence

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To its mouse counterpart (Supplementary Fig. 1A). In mice, ALG-2 protein showed 52% amino acid identity and 61% similarity to cell receptors, Fas and glucocorticoid [9]. The putative ALG-EF-hand protein required for T-cell apoptosis induced by T ALG-2 forms a complex with ALIX in a calcium-dependent manner [12]. Moreover, ALG-2 and ALIX form a complex with SETA/CIN85, an SH3-containing protein, and modulate cell death and endosomal trafficking [17], suggesting that POSH may form a complex with ALG-2 via a Drosophila homolog of ALIX.

We cloned a cDNA encoding a homolog of ALIX in Drosophila. The amino acid sequence of the putative Drosophila ALIX protein deduced from the cDNA sequence showed 41% identity and 61% similarity to mouse ALIX, and contained a proline-rich region in the C-terminal half (Supplementary Fig. 1B).

3.2. POSH forms a complex with ALG-2 and ALIX

GST pull-down assays were performed to investigate whether POSH formed a complex with ALG-2 and ALIX using proteins synthesized in vitro. Bacterially-expressed GST–ALG-2 fusion protein alone failed to pull down POSH protein in either the presence or absence of calcium (data not shown). However, the GST–ALIX fusion protein was able to bind to POSH (Fig. 1A). Furthermore, the GST–ALIX protein was also able to bind to ALG-2, but only in the presence of calcium.

3. Results

3.1. Identification of ALG-2 and ALIX as POSH-interacting proteins

To identify POSH-interacting proteins, we performed yeast two-hybrid screening using a full-length POSH cDNA fused to the GAL4 DNA binding domain as bait. Approximately 6 x 10^8 clones in a Drosophila adult cDNA library were screened, and 12 clones that activated the β-galactosidase reporter gene were isolated. Sequence analysis of the library inserts revealed two independent clones that contained an open reading frame of a Drosophila homolog of ALG-2, a penta-EF-hand protein required for T-cell apoptosis induced by T cell receptors, Fas and glucocorticoid [9]. The putative ALG-2 protein showed 52% amino acid identity and 61% similarity to its mouse counterpart (Supplementary Fig. 1A). In mice, ALG-2 forms a complex with ALIX in a calcium-dependent manner [12]. Moreover, ALG-2 and ALIX form a complex with SETA/CIN85, an SH3-containing protein, and modulate cell death and endosomal trafficking [17], suggesting that POSH may form a complex with ALG-2 via a Drosophila homolog of ALIX.

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of calcium (Fig. 1B). To identify the binding domain, we performed separate GST-pull down assays with the N-terminal and C-terminal parts of the ALIX protein. The C-terminal half of ALIX bound to both GST–POSH and GST–ALG-2, whereas the N-terminal half did not bind to either GST fusion protein. These results indicate that the C-terminal half of ALIX containing the proline-rich region is responsible for the binding to both POSH and ALG-2 (Fig. 1C).

Next, we examined the interactions among these proteins in cultured Drosophila S2 cells. Lysates from S2 cells co-transfected with different combinations of the expression constructs were immunoprecipitated with an anti-POSH antibody, and subjected to immunoblot analyses with anti-ALG-2 or anti-ALIX antisera. Both ALG-2 and ALIX co-precipitated with the anti-POSH antibody (Fig. 1D). Based on the GST-pull down experiments described above, ALIX is likely to bind directly to both POSH and ALG-2. Taken together, these results suggest that POSH, ALG-2 and ALIX form a complex in the cultured cells.

3.3. Expression of ALG-2 and ALIX

Northern blot analysis revealed that ALG-2 and ALIX were expressed throughout development and in S2 cells as 0.5 and 3 kb transcripts, respectively (Fig. 2A). In situ hybridization was performed to determine the spatiotemporal patterns of the transcript expressions during embryogenesis and larval development. Both the ALG-2 and ALIX transcripts were predominantly detected in the nervous system in early stage embryos (Fig. 2C). Thereafter, both transcripts were expressed ubiquitously in embryos and imaginal discs (Fig. 2B and C). The immunostaining patterns using anti-ALG-2 and anti-ALIX antisera were consistent with the RNA localizations (data not shown).

3.4. Overexpression of ALG-2 and ALIX induces morphological defects

Next, we examined the effects of overexpression of ALG-2 and ALIX using transgenic flies bearing UAS-ALG-2 or UAS-ALIX. Overexpression of each of these genes individually or in combination with UAS-POSH was induced in developing eye imaginal discs using GMR-GAL4. Overexpression of ALG-2 induced a rough eye phenotype, which was similar to that induced by POSH overexpression [7]. Flies overexpressing both ALG-2 and POSH had small eyes (Fig. 3A–C), suggesting that cell death occurs during development. Overexpression of ALIX resulted in a partial loss of the red pigments and produced melanotic pigments, suggesting neuronal degeneration. The phenotype induced by ALIX overexpression was enhanced by co-overexpression of POSH, which increased the amount of melanotic pigments but did not affect the eye size (Fig. 3D–F). These results suggest that both ALG-2 and ALIX, in combination with POSH, could kill the cells during development, but this effect is more efficient for ALG-2/POSH than for ALIX/POSH.

3.5. Overexpression of ALG-2 and ALIX activates the JNK pathway

Finally, we examined whether overexpression of ALG-2 and ALIX could activate the JNK pathway, puc, encoding a dual-specificity phosphatase, is a target of the JNK pathway. We used a puc-lacZ enhancer trap line to monitor the JNK signaling activity by the LacZ expression level [15,16]. Overexpression of ALG-2 or ALIX in imaginal wing discs using ptc-GAL4 induced ectopic JNK activation along the border of the anterior/posterior compartments. Consistent with this, overexpression of ALG-2 and ALIX disrupted the orientation of the wing hairs (Fig. 4D and E, data not shown), similar to...
the case for POSH overexpression [7]. These results demonstrate that overexpression of ALG-2 and ALIX, as well as POSH, can activate the JNK signaling pathway, which may lead to various biological processes, including cell death.

4. Discussion

In the present study we demonstrated that POSH can form a complex with ALG-2 and ALIX both in vitro and in Drosophila cultured cells. Transgenic studies revealed that overexpression of ALG-2 in imaginal eye discs using GMR-GAL4 resulted in a roughened eye, similar to that induced by POSH. However, when both ALG-2 and POSH were overexpressed, the eye size was dramatically reduced, suggesting that ALG-2 in combination with POSH could kill the cells during development. Overexpression of ALIX produced eyes with melanotic pigments, which was indicative of neuronal degeneration. Flies overexpressing both ALIX and POSH showed increased amounts of melanotic pigments, with no effect on the eye size. The phenotypic differences between ALG-2 and ALIX suggest that the cell death occurs more rapidly in eye discs overexpressing ALG-2/POSH than in those overexpressing ALIX/POSH.

We further demonstrated that ALG-2 and ALIX can activate the JNK signaling pathway, similar to the case for POSH. Since neither ALIX nor ALG-2 has a putative kinase domain, overexpression of either protein together with POSH may facilitate ectopic JNK activation by affecting the subcellular localization of JNK components. In fact, ALG-2 has been shown to interact with ASK1, thereby regulating its subcellular localization and JNK activation in mammalian cells [18, 19].

ALG-2 and ALIX are both thought to be involved in membrane trafficking [20–22]. ALG-2 interacts with Annexin XI and VII, both of which play roles in vesicular trafficking and exocytosis [23]. On the other hand, ALIX binds to CHMP4b, a human homolog of yeast Snf7, which is involved in multivesicular body (MVB) sorting [20]. Furthermore, ALG-2 and ALIX both bind to Tsg101, a component of ESCRT-I. ESCRT-I cooperates with two other complexes, ESCRT-II and ESCRT-III, to drive MVB formation [21, 22, 24]. MVB sorting is thought to be topologically identical to the budding of HIV and other retroviruses from the plasma membrane. Indeed, ALIX has been shown to associate with HIV-1 Gag protein, which is required for promoting membrane fission events, and recruit the ESCRT machinery to permit budding [21, 22]. Recently, POSH was also shown to be required for sorting of HIV-1 Gag protein to the plasma membrane. Furthermore, POSH interacts with hepatocyte growth factor-regulated tyrosine kinase substrate, which is known to play a central role in MVB formation, and modulates its stability [25]. These findings suggest that the POSH/ALIX/ALG-2 complex may have a role in membrane trafficking and virus budding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.05.005.
References


