OSBPa, a predicted oxysterol binding protein of *Dictyostelium*, is required for regulated entry into culmination

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Abstract The oxysterol binding proteins (OSBPs) are believed to control cholesterol homeostasis but their precise mechanism of action is not well understood. The *Dictyostelium osbA* gene encodes a predicted OSBP, OSBPa, which lacks the PH domain that in most other OSBPs directs targetting to the Golgi. OSBPa instead localises selectively to the cell periphery and also, in some cells, to the perinuclear region. OSBPa null strains form normal fruiting bodies but are defective in the regulation of the transition from slug migration to culmination. Thus a plasma membrane-enriched OSBP family member is essential for correct regulation of the slug–fruiting body switch. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Oxysterol binding protein; 'Slugger' mutant; Culmination; *Dictyostelium discoideum*

1. Introduction

When oxysterol binding proteins (OSBPs) bind oxidised forms of cholesterol they translocate from the cytoplasm to the Golgi [1,2]. In mammalian cells OSBP function is not well understood but the best characterised family member, OSBP1, is believed to modulate sterol and phospholipid synthesis [3,4]. In yeast the best characterised OSBP, Kes1P, regulates the Sec14p Golgi-derived vesicle transport pathway [5]. Kes1P is unusual in that it does not possess a PH domain. Most members of the OSBP protein family contain such a domain and both a yeast and a mammalian OSBP-derived PH domain have been shown to direct Golgi targetting [2].

When *Dictyostelium* cells are subjected to starvation they aggregate together and form themselves into a patterned structure, the fruiting body, comprised of a mass of spores surmounting a stalk. Under appropriate developmental conditions a migratory slug is formed, with prestalk cells in its front one-fifth and prespore cells at the rear. The prestalk region can be sub-divided, using cell type-specific markers. The prestalk O (pstO) cells form a band, occupying the rear two-thirds of the prestalk region, and the pstA cells occupy the front one-third [6]. We describe a protein, OSBPa, that is marginally enriched in pstO cells, that is a member of the OSBP family of proteins and that, unusually for OSBP proteins, is selectively localised at or near to the plasma membrane.

2. Materials and methods

2.1. Cell culture and manipulation

The Ax2, axenic derivative of NC4 was used throughout and it was cultured axenically at 22°C. Transformation was performed by electroporation and strains were selected at 10 μg/ml of blasticidin S. For development, cells growing at a density equal to or less than 2×10^6/ml were washed twice in KK2 phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2). They were allowed to develop on nitrocellulose filters lying on a KK2-soaked pad at 22°C in a weak unidirectional light source.

2.2. Antibody preparation and histochemistry

A rabbit polyclonal antibody was raised against a peptide sequence derived from the predicted N-terminus of OSBPa (MGKKDKNVSVEEEVDC). The antibody was affinity-purified using the peptide immunogen [7] and was employed for both Western transfer analysis and histochemical staining. Slug whole mounts were prepared by floating the slugs, off the plate, in methanol at −20°C and incubating them in methanol at −20°C for 5 min. For intracellular localisation of OSBPa, slugs were dissociated into small cell clumps in KK2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2), by repeated passage through a plastic pipette tip. The cell clumps were fixed with methanol at −20°C and attached to a glass slide. Whole mount and dissociated specimens were stained with affinity-purified anti-OSBPa antibody using an Alexa Fluor 594-conjugated, anti-rabbit IgG second antibody (Molecular Probes). In some experiments double staining with OSBPa was performed using an anti-comitin antibody. Cells were dissociated from slugs, fixed with methanol at −20°C and double-stained with anti-OSBPa and a mouse monoclonal anti-comitin antibody [9]. OSBPa was detected using Alexa Fluor 594-conjugated anti-rabbit antibody and comitin was detected using Alexa Fluor 488-conjugated anti-mouse antibody (both from Molecular Probes). Images were obtained using a Leica TCS confocal microscope with excitation wavelengths of 488 (for Alexa Fluor 488), and 543 (for Alexa Fluor 594), yielding non-overlapping emission wavelengths of 500–525 (for Alexa Fluor 488) and 580–640 (for Alexa Fluor 594). Bar, 5 μm.

2.3. Cell fractionation

Slug cells were lysed by forcing them through a nucleopore filter (pore size 3 μm) at 5×10^8 cells/ml in lysis buffer (10 mM Tris–HCl pH 8.0, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride). Nuclei were collected by centrifugation at $3000\times g$ for 2 min. The supernatant was further centrifuged at $15\,000\times g$ for 5 min to pellet plasma membranes. The membrane and nuclear fractions were washed once with lysis buffer and were resuspended in lysis buffer at 1/10th of the original volume of the lysate. The final supernatant was used as a cytosolic fraction.

2.4. Disruption of the OSBPa gene

An OSBPa gene fragment, containing a blasticidin resistance cas-

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Abbreviations: OSBP, oxysterol binding protein; pstO, prestalk O

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MGKKDKNVSVEEEVDEAEIEKLAAENANKPAPQLTKEDLD   40
AMDDAEPGTSRLALIGKMIKKVSIGTDISNISMPGSFILA   80
KSTLSYFSDNFSSFFGELLKANKIDNELERMLQVQRYLFT  120
TLKETEDTTRKPLNPILGETWQANIHVKDGDGNDVTDNYF  160
FAEQISHHPPISSSTVYNKKEGVNCTFCLPVRSQFMGTYV  200
KISFEGESHITFEKYDEKFTFVAPPMAIRIFRSFSEYVGK  240
GILKSDKNDYLIKSTYTSKPLFGGVYNGFESKVYKGKEKL  280
YKIKGTWSGDMKITNLKTNETTPFFTRPTESAKVVFPDDG  320
NTLPTDSSVVWKGVFDASKKDDVKGMSQEKVKVEEEQRKL  360
AHHRKNADEWKPVHFNKIDGRWQLVNLKD             389
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Fig. 1. The predicted amino acid sequence of OSBPa. The highly conserved SHHPP motif that typifies OSBPs is shown in bold letters and underlined. The accession number of the *osbA* cDNA sequence is AJ488770.

sette, was cloned into pBluescriptII and the *Eco*RI–*Kpn*I fragment (see legend to Fig. 5A) was used for transformation and gene disruption of *Dictyostelium*.

3. Results

3.1. The osbA gene encodes an OSBP homologue that lacks a PH domain

The gene encoding OSBPa, *osbA*, was isolated in a yeast two-hybrid screen using the *Dictyostelium* Dd-STATc gene [8] as bait. Although subsequent pull-down experiments confirmed an in vitro interaction, genetic studies have thus far failed to uphold a role for OSBPa in Dd-STATc function. Nonetheless, we continued our analysis of OSBPa, because the gene inactivation studies showed that OSBPa is a developmental regulator. The sequence of the full-length cDNA clone (Fig. 1) predicts it to encode a member of the OSBP family of proteins. OSBPa resembles proteins such as the HES and KES1 protein of *Saccharomyces cerevisiae* in lacking a PH domain but it contains the SHHPP motif that defines the OSBP family and significant addition homology (Fig. 2A). A search of the database of the *Dictyostelium* genomic sequence (http://www.sanger.ac.uk/Projects/D_discoideum/) shows that there are at least four additional 'small' OSBP genes within the *Dictyostelium* genome (Fig. 2B). At the time of searching, the contig sequence data were estimated to be 90% complete, i.e. there was a 90% chance that any search sequence would have been present within the contig set (M. Rajandream, personal communication).

3.2. The OSBPa protein accumulates during multicellular development and is enriched in a cytosolic fraction

A polyclonal, affinity-purified antibody was prepared against an N-terminal peptide from OSBPa. When used to stain a Western transfer (Fig. 3A) the antibody detects a protein with approximately the estimated size of OSBPa (44 kDa). OSBPa is strongly developmentally regulated in its accumulation, reaching peak concentration during culmination. When cells are lysed by passage through a nucleopore membrane, and fractionated by differential centrifugation, OSBPa is highly enriched in the cytosolic fraction (Fig. 3B). There is also a significant amount of OSBPa in the nuclear fraction but there are only minor amounts in the membrane fraction.

3.3. Immunochemical analysis of OSBPa shows that it is slightly enriched in pstO cells and predominantly concentrated at the periphery of the cell

Immunostaining of whole mounts of slugs shows that OSBPa is somewhat enriched in the pstO cells (Fig. 4A) and also in the rearguard cells: a population of prestalk cells, seen at the rear of some slugs, that form part of the basal disc at culmination (data not shown). This is, however, only a small, quantitative difference and there is considerable staining in the pstA and prespore regions. This is confirmed using cells partially dissociated from slugs. All cells in such clumps are strongly stained with the OSBPa antibody (Fig. 4B). Staining in dissociated cells is predominantly localised to the cell periphery but in some cells there is also perinuclear OSBPa staining (arrowed in Fig. 4B). In order to determine whether this latter staining is to the Golgi, an anti-comitin antibody (comitin is a well-characterised Golgi marker [9]) was used in double staining with the OSBPa antibody. These data show that OSBPa is not significantly enriched in the Golgi (Fig. 4B, merged image).

3.4. Inactivation of the osbA gene leads to a 'slugger' phenotype

A disruption construct was created (Fig. 5A), to inactivate the *osbA* gene, and introduced into *Dictyostelium* cells by transformation. Five independent disruptant clones were identified by PCR analysis and this was confirmed by Western transfer analysis (Fig. 5B). Cells from all five clones grow normally and produce correctly proportioned fruiting bodies. However, when growing on a bacterial lawn, they produce very characteristic plaques in which fruiting bodies are present within the bacterial lawn outside the plaque rather than, as normal, only within the plaque confines. Also, they are all sluggers. This term denotes a strain where aggregates form migratory slugs under developmental conditions that would cause parental structures to culminate [10,11]. The parental Ax2 strain almost always forms fruiting bodies in situ, with no migratory slug phase, when cells are developing on a nitrocellulose filter (Fig. 5C). In contrast, OSBPa null cells readily form migratory slugs when developing on a nitrocellulose filter (Fig. 5C shows one of the five disruptant clones). Although slugs of the OSBPa strain are defective in regulation of the migration vs culmination switch, they show normal phototaxis and their behaviour after they commit to culmination is normal (data not shown).

4. Discussion

Although most interest has been focussed on the role of OSBPs in lipid metabolism and vesicle trafficking there are indications that they might fulfil other functions. OSBP-Dm is a *Drosophila* OSBP protein [12]. The existence of a *Drosophila* OSBP homologue is in itself intriguing, given their supposed function in mammals, because insects are unable to synthesise a sterol core. They instead rely upon sterols in their diet. Furthermore, the OSBP-Dm protein was identified not as a biosynthetic regulator but as a suppressor of the cell cycle arrest induced when the Wee1p regulator is over-expressed in fission yeast. The BRAM protein of *Xenopus laevis* interacts, physically and functionally, with the transforming growth factor β (TGF-β) superfamily member BMP [13]. BIP is an OSBP family member that interacts with BRAM and genetic

evidence suggests that the nematode BIP and BRAM homologues form part of a TGF-β signalling pathway that regulates body length.

In common with the BIP proteins and a number of other OSBP family member proteins, OSBPa does not possess a PH domain. This may explain why it is not enriched in the Golgi; because the PH domains of a mammalian and a yeast OSBP have both been shown to act as Golgi-targetting domains [2]. OSBPa is instead selectively enriched at cell periphery. It is not, however, an integral plasma membrane protein nor can it be tightly associated with the plasma membrane, because after cell fractionation it is much more highly enriched in the cytosol than in the membrane fraction. Cell staining reveals the presence of perinuclear OSBPa in some cells. This is consistent with the cell fractionation analysis, because the nuclei were prepared without detergent treatment and are therefore contaminated with perinuclear material. The significance of this perinuclear material is, however, unclear.

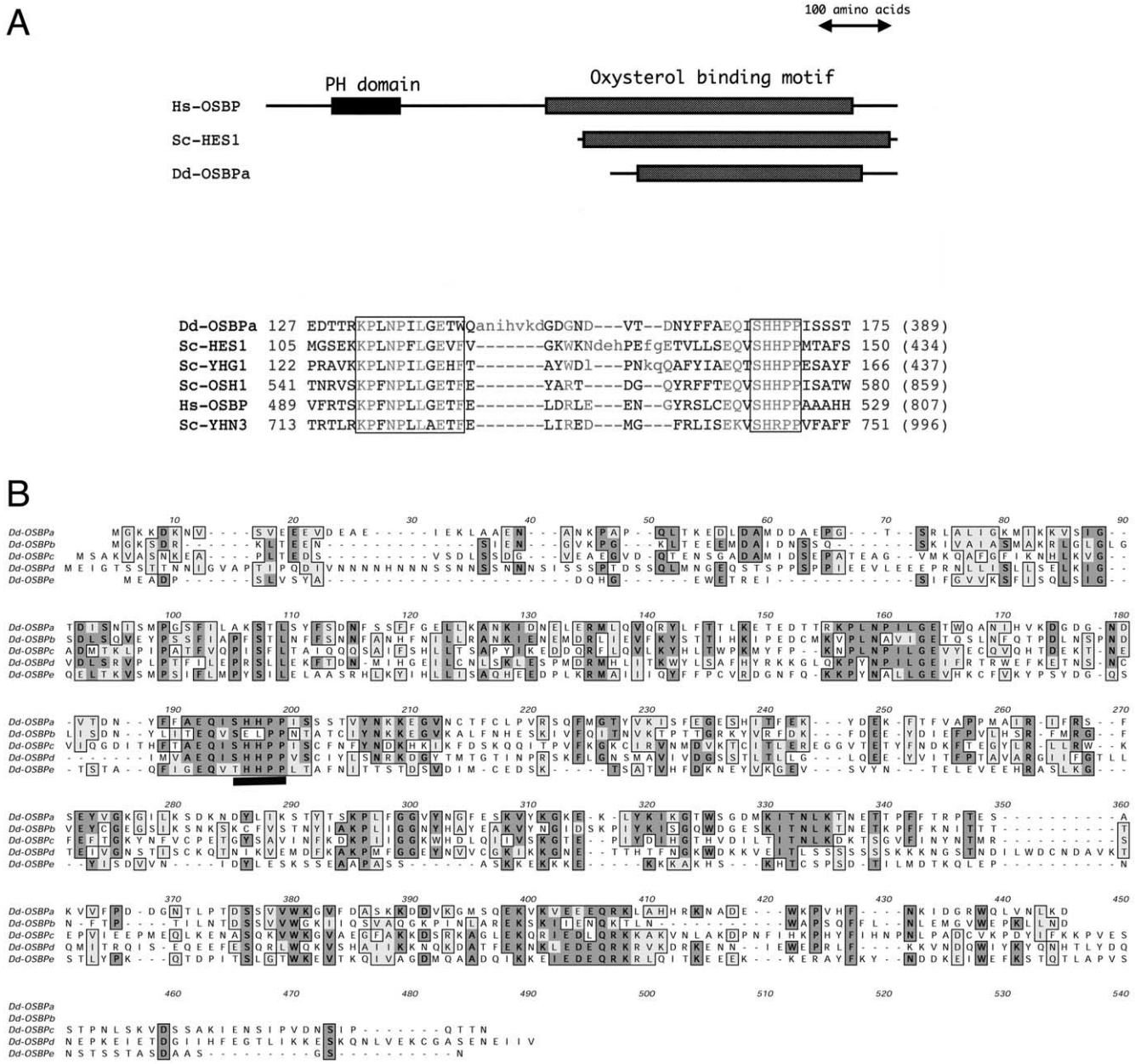

Fig. 2. A: Schematic of the domain structures of human and yeast OSBPs and of Dd-OSBPa and an alignment of the most highly conserved region of Dd-OSBPa with mammalian and yeast OSBPs. The upper part of this figure shows schematic OSBP structures; the archetypal member of the family, human OSBP1, contains a PH domain, as do most mammalian OSBPs. OSBPa of *Dictyostelium* resembles *S. cerevisiae* proteins such as HES1, in lacking a PH domain. In the lower part of the figure, the sequence motif that most closely defines OSBP family members, SHHPP, is indicated by a box. Another highly conserved upstream region is also boxed. B: Identification of four predicted 'small' OSBP genes in the *Dictyostelium* genome. Contigs assembled at the Sanger sequencing centre, from data generated by the International *Dictyostelium* Genome Sequencing Consortium, were searched using OSBPa and four additional small OSBP family members (i.e. OSBPs that lack a PH domain) were identified. At the time of searching, the contig sequence data were estimated to be 90% complete, i.e. there was a 90% chance that any search sequence would have been present within the contig set (M. Rajandream, personal communication). The sequence motif that defines OSBP family members, SHHPP, is underlined.

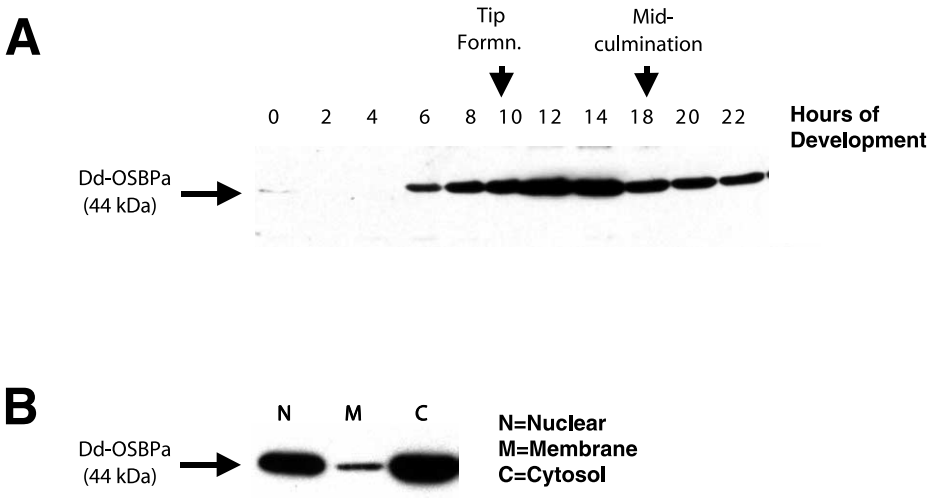

Fig. 3. A: Western transfer analysis of the accumulation of OSBPa during development. Total cell lysates prepared from the indicated developmental stages were subjected to Western transfer analysis using an affinity-purified anti-OSBPa antibody. B: Western transfer analysis of OSBPa in fractionated cell extracts. Slug cells were mechanically lysed and fractionated into nuclear, cytosolic and membrane fractions by differential centrifugation. Aliquots, each containing 5×10^6 cell equivalents, were subjected to Western transfer analysis using affinity-purified anti-OSBPa antibody.

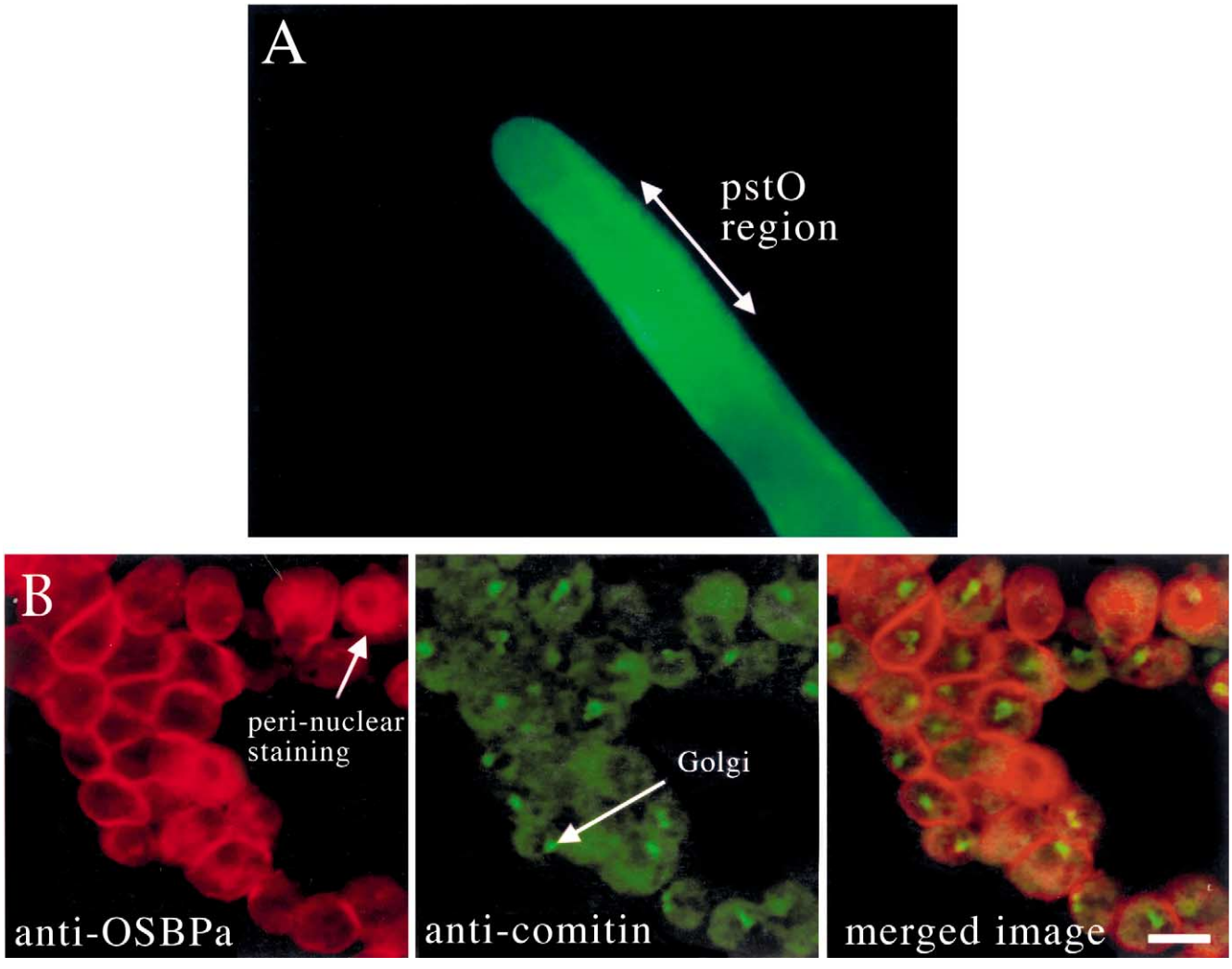

Fig. 4. A: The distribution of OSBPa in whole mount slug preparations. Slugs were fixed with methanol at −20°C and stained with an anti-OSBPa antibody as described in Section 2. This is a low power image of the front part of a slug showing that the OSBPa protein is marginally enriched in the pstO region. B: The localisation of OSBPa and comitin in dissociated slug cells. Cells dissociated from slugs were double-stained for OSBPa and comitin as described in Section 2.

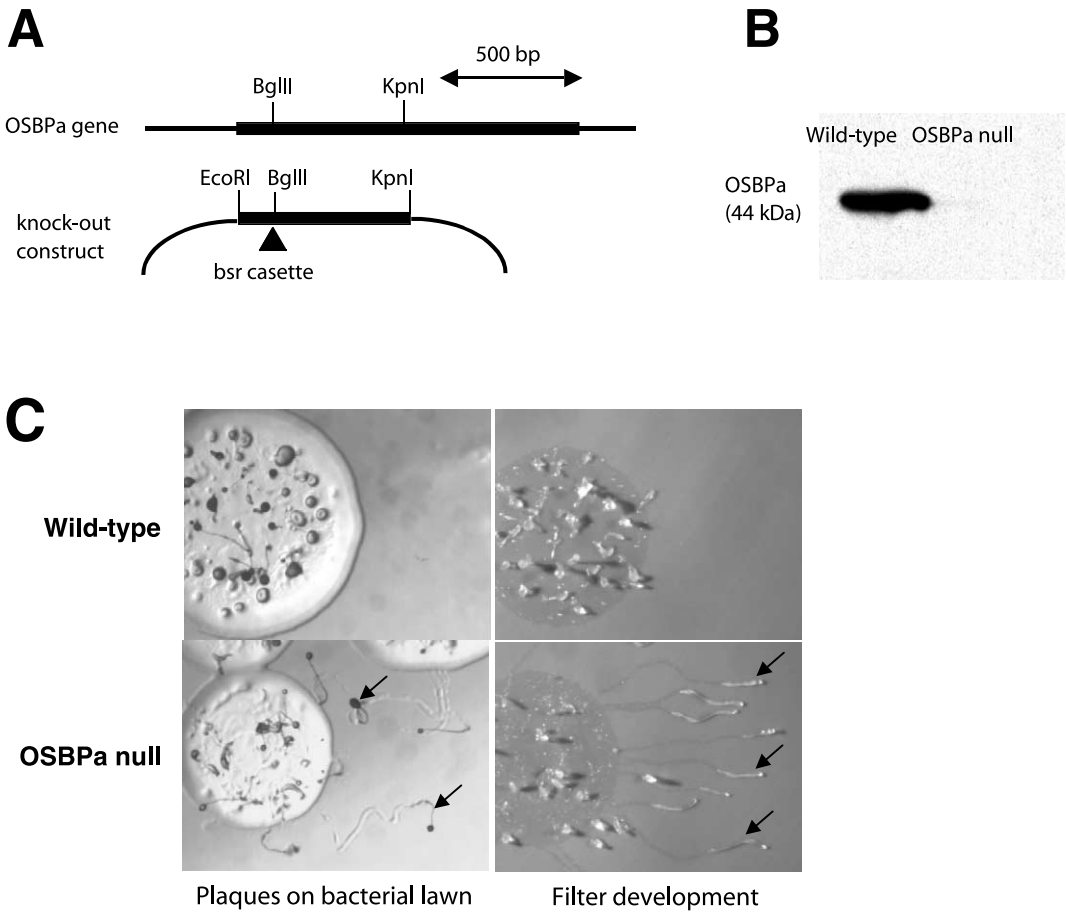

Fig. 5. A: Structure of the OSBPa gene and of the OSBPa knockout construct. In order to create a knockout construct, the OSBPa coding region was PCR-amplified as an *Eco*RI–*Xho*I fragment and cloned into the pBluescriptII vector (Stratagene). A blasticidin-resistant (bsr) cassette of 1.5 kb was then cloned into a *Bgl*II site, located near the ATG start codon. B: Western transfer analysis of the OSBPa null strain. Parental or OSBPa null cells were allowed to develop for 16 h on a water agar plate and lysed samples of 2×10^6 cells were subjected to Western transfer analysis. C: Analysis of plaque morphology and of the switch to culmination in an OSBPa null strain. When growing on a bacterial, *Klebsiella aerogenes* lawn OSBPa null strains display a characteristic plaque morphology. The plaque is smaller than for the parental strain and fruiting bodies arise beyond the periphery of the plaque, within the bacterial lawn (indicated by arrows). When developing on a nitrocellulose filter parental structures culminate in situ, with no migratory slug phase. In contrast, OSBPa null structures migrate as slugs away from the site of deposition (the slugs are indicated by arrows).

Development of OSBPa to form a fruiting body is outwardly normal. However, OSBPa is important in ensuring correct regulation of the slug–fruiting body switch. Similar mutants defective in regulation of the entry into culmination, slugger mutants, were originally identified using classical genetic methods [10,11]. Subsequently other genes have been identified that result in a slugger phenotype when disrupted [8,14,15]. This is a rather subtle phenotype but there is the possibility that OSBPa may be partially redundant with another OSBP. There are at least four similarly structured OSBPs in the genome that could fulfil such a semi-redundant role.

Given the way that OSBPa was identified, it is perhaps of note that null mutants for Dd-STATc, the gene used in the yeast two-hybrid screen, are sluggers, with a similar plaque morphology and that Dd-STATc is nuclear localised selectively in the pstO region [8]. However, the fact that in the OSBPa null strain Dd-STATc remains fully responsive to its inducer, the chlorinated hexaphenone DIF (Fukuzawa and Williams, unpublished data), argues against a role of OSBPa in the functioning of Dd-STATc. Further analysis, using techniques such as gene expression profiling, will be required to determine the precise role that OSBPa plays in regulating the slug–fruiting body switch.

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