The *Aspergillus niger* annexin, *anxC3.1* is constitutively expressed and is not essential for protein secretion

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Abstract

An annexin, *anxC3.1*, was isolated and characterised from the industrially important filamentous fungus *Aspergillus niger*. *anxC3.1* is a single copy gene encoding a 506 amino acid predicted protein which contains four annexin repeats. Disruption of the *anxC3.1* gene did not lead to any visible changes in phenotype, nor in the levels of secreted protein, nor specifically in glucoamylase production, suggesting no major role in secretion. *anxC3.1* expression was found to be unaltered under a variety of conditions such as increased secretion, altered nitrogen source, heat shock, and decreased Ca\(^{2+}\) levels, indicating that *anxC3.1* is constitutively expressed. This is the first reported functional characterisation of a fungal annexin.

Keywords: Annexin; Secretion; *Aspergillus niger*; Gene disruption

1. Introduction

The annexins are a large family of structurally related proteins that exhibit the common features of Ca\(^{2+}\) and phospholipid binding. At least 27 members of this family have been described, with annexins identified in many mammalian species, as well as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Dictyostelium discoideum*, *Hydra vulgaris*, *Giardia lamblia* and a number of plants including strawberry, celery and garden pea [1,2]. Although there is no annexin homologue in the yeast *Saccharomyces cerevisiae*, Braun et al. [3] identified an annexin in the filamentous fungus *Neurospora crassa* by searching of ESTs followed by sequence and phylogenetic analyses. We have recently identified annexins in *Aspergillus fumigatus* and *A. nidulans* [4].

The molecular basis of Ca\(^{2+}\) and phospholipid binding by the annexin repeats is well established [5,6], however, the in vivo function of annexins in animals is still far from clear. In mammals, annexins have been implicated in a number of membrane events including regulated and constitutive exocytosis, endocytosis and membrane organisation [7–9]. In plants, a role for annexins in membrane trafficking and/or secretion, calcium channel formation and calcium hemostasis has been suggested [10–13].

To date, nothing is known about the function of fungal annexins. In filamentous fungi, growth is achieved...
by a polarised extension of the hyphal tip. The new plasma membrane and cell wall materials are delivered to the site of growth by Golgi apparatus-derived secretory vesicles [14–16]. Tip growth has been correlated with a localised high Ca$^{2+}$ gradient and a number of studies have implicated Ca$^{2+}$ in hyphal growth regulation in fungi [17–20]. Based on these data and those for animal and plant annexins, fungal annexins may have a role in membrane events and calcium regulations during exocytosis and tip growth.

In the present study we have isolated and characterised an annexin gene from A. niger. We analysed its expression pattern and showed the effect of gene deletion.

2. Materials and methods

2.1. Strains and plasmids

A. niger strain N402 [21] and a pyrG derivative, AB4.1 [22] were used for isolation of an annexin homologue and for the annexin disruption experiments. Escherichia coli TOP 10 (Invitrogen) cells were used in transformation experiments. pGEM® T Easy cloning vector (Promega) was used for cloning of PCR products and plasmid Litmus™ 28 (New England Biolabs) was used for the plasmid libraries.

2.2. Growth, culture conditions and fungal transformations

Modified Vogel’s medium [23] (modified by substitution of 10 g/l maltodextrin, glucose or xylose for sucrose) was used for fungal growth experiments and supplemented with uridine/uracil when needed. Liquid cultures were grown at 30 °C in 250 ml flasks and agitated at 225 rpm. Transformation of A. niger protoplasts was carried out using standard PEG transformation method [24].

2.3. DNA and RNA procedures

Fungal DNA and RNA samples were prepared using commercial kits (Q.Biogene). Molecular methods for Southern analysis, restriction enzyme analysis, ligation of DNA fragments and transformation of E. coli were performed essentially as described by Sambrook et al. [25]. For Reverse transcriptase-PCR reactions, Prostar™ HF single tube RT-PCR kit (Stratagene) was used.

2.4. Cloning of the A. niger annexin, anxC3.1

The degenerate primers VK12 (5’-GG N TAY TAY CAR CAR CCN CC-3’) and primer VK14 (5’-YTC RTC NGT NCC CCA N CC YTT CAT-3’), based on the N. crassa annexin cDNA sequence (GenBank Accession No., AF036871) were used with the following cycle conditions; 30 cycles of 2 min at 94 °C, 1 min at 56 °C, 1.5 min at 72 °C. The 195 bp fragment produced was used to clone a 1.4 kb EcoRI/BamHI fragment from a size-restricted plasmid library of A. niger genomic DNA in pLitmus28™. A second size restricted library containing ~3 kb SacI genomic fragments was screened using the 1.4 kb EcoRI/BamHI fragment as a probe (see above). A 3 kb fragment containing the entire gene plus 5’ and 3’ untranslated regions was subsequently identified.

2.5. Construction of deletion cassette

An in vitro transposition system was used to make the annexin disruption cassette. The A. fumigatus pyrG gene along with its own promoter and terminator was cloned into the EcoRI site of EZ::TN™ pMOD2™-2<MSC> Transposon Construction Vector (Epicenter, Madison, USA). The Zeocin resistance gene, under the control of bacterial EM7 promoter, was excised from pEM7/Zeo (Invitrogen) using an XbaI/NheI double digest and cloned into XbaI site of pMOD2-pyrG to give plasmid pMOD2-pyrGZeo, thereby providing a bacterial selection marker. The pyrGZeo transposon was then excised from pMOD2-pyrGZeo using PshAI enzyme and gel purified, resulting in a ~2.4 kb fragment. The purified transposon was used to disrupt the anxC3.1 gene which had already been cloned in pLitmus28 as an ~3 kb genomic fragment. The transposition reaction was carried out using transposase (Epicenter, Madison, USA) according to the manufacturer’s instructions. Resulting transposed anxC3.1 were screened by PCR for transposition inside anxC3.1; forward primer ANXC3.1_F1(5’-TCC GCC ACA GGG CTA TAA TC-3’) and reverse primer ANXC3.1_R1(5’-CAA ACG GCC ATC ATT GCA T-3’); reaction conditions, 30 cycles of 2 min at 94 °C, 1 min at 55 °C, 3.5 min at 72 °C. The primers correspond to nucleotides 30–1513 of the anxC3.1 ORF, resulting in an ~3.9 kb product in disrupted plasmids. Plasmid contained disrupted anxC3, named pAX-KO, was selected on the basis of further PCRs using primer pairs spanning the mosaic end of the transposon and the start of the gene, confirming that the transposon was in the middle of anxC3.1. A 10 µg aliquot of pAX-KO was digested with AgeI and AflII and a resulting fragment of ~5.6 kb containing the disrupted anxC3.1 construct was purified.

2.6. Total secreted protein and glucoamylase assays

Fungal cultures were grown in modified Vogel’s medium containing maltodextrin, as described above and supernatants were sampled after 18 h of culture. Total secreted protein was determined by means of the
Bradford assay and normalised for dried biomass. The glucoamylase assay was carried out in microtitre plates as follows: To each well was added 15 μl supernatant and 130 μl substrate (1 mg/ml p-nitrophenol-α-D-glucopyranoside in 0.54 M sodium acetate pH 4.3). Samples were incubated for 30 min at 37 °C after which reactions were stopped by the addition of 160 μl Na2CO3. Plates were then read at 400 nm. The amount of p-nitrophenol produced was calculated and values were normalised for the amount of dried biomass.

3. Results and discussion

3.1. Isolation of the A. niger annexin gene

The full-length annexin gene was cloned as a 3 kb fragment which contained a single 1.5 kb ORF encoding a protein of 506 amino acids with a calculated molecular mass of 55.8 kDa, plus 5′ and 3′ flanking sequence (Gene Bank Accession No. AY033935.1). Southern analyses showed that this gene was present as a single copy in the A. niger genome (data not shown). We recently reported the identification of two annexins, ANXC3.1 and ANXC3.2 in A. fumigatus [4].

The A. niger annexin reported here is similar to both of these, but resembles ANXC3.1 more closely (53% identity, compared to 37% identity to ANXC3.2; Fig. 1). We have named the gene annexin C3.1. in line with the annexin classification established by Morgan and Fernandez [26,27].

3.2. Gene and protein structure

The 200 amino acid N-terminal tail on ANXC3.1 was longer than the comparable region from A. fumigatus, N. crassa, D. discoideum or human annexins (Fig. 1). However, this region shared with these annexins a biased amino acid composition with proline (30%) glutamine (22.5%) glycine (14%) and tyrosine (10.5%) predominating. The unusual composition of the ANXC3.1 N-terminal tail is seen in annexin XIV and annexin VII proteins. Since the N-terminal tail of human annexin 7 interacts with sorcin [28], it is most likely that this region of annexin C3.1 is involved in interactions with other similar proteins. Significantly, blast searches against fungal genome databases using human sorcin as the query sequence identified sorcin homologs in various filamentous fungi. In each case only a single homolog was present (unpublished data).

The calcium-binding repeat regions I and II of the predicted ANXC3.1 protein were similar to those seen in annexin VII proteins with conservation of the MXG motif in the MKGFGTDE sequence in repeat I and MSGLGTNE sequence in repeat II (see Fig. 1). Repeats I and II also show the presence of the conserved glutamate residues approximately 40 amino acids downstream of the MxG motif, following the consensus sequence of GXGT...(D/E). The degree of homology is less for the fourth repeat, especially in the calcium coordinating residues. These differences also have been observed in plant annexins [12].

The relationships between annexins from different species and kingdoms has been assessed in a number of papers [1,3,4,27]. The fungal annexins are monophyletic, and although they cluster with the animal sequences, this is with poor bootstrap values. The fungal annexins do not consistently cluster with one set of animal annexins and may have diverged before the proliferation of annexins seen in the animal kingdom. Although this does not provide evolutionary support for the similarity between the N-terminal tails of annexin VIIIs and fungal annexin C3s, it is still conceivable that similar sequences were present in ancestral animal annexins, but have been subsequently lost in most members as the animal annexin family expanded.

3.3. The anxc3.1 gene is not essential for growth or secretion

A. niger pyrG Protoplasts were transformed with Agel II fragment of pAX-KO. Screening of fungal transformants by PCR identified a transformant with homologous gene replacement (Fig. 2(a)). Southern analysis using Sall and HindIII genomic digests probed with an xc3.1, confirmed homologous replacement with the disrupted anxc3.1 gene (Fig. 2(b)).

Examination of growth rates and phenotypes indicated that there was no significant difference in radial growth rates between the anxc3.1 disruptant and the wild-type parent strain when grown on modified Vogel’s, using different carbon sources including glucose, maltodextrin and xylose and different nitrogen sources like ammonium nitrate, ammonium sulphate and peptone, or on complete medium at 30, 37 or 42 °C (data not shown).

Annexin proteins require calcium for their biological function and therefore the growth and phenotype of the anxc3.1 disruptant was tested on Vogel’s medium containing the calcium chelator 1,2-bis[2-aminophenoxy] ethane-N,N,N′,N′-tetra acetic acid (BAPTA, 1 mM) (data not shown). No difference was observed between wild type strain and disruptant. These results indicated that anxc3.1 is not essential for the growth of A. niger under the conditions tested.

The effects of anxc3.1 disruption on protein secretion were also examined. Measurement of the amount of total secreted protein showed that the knockout produced 9.38 ± 0.57 mg/l total protein (n = 3) while the parent strain produced 8.72 ± 0.67 mg/l (n = 3), i.e. there was no significant difference in the amount of total secreted protein. Examination of glucoamylase levels demonstrated that the parent strain produced 7.42 ± 0.69 μM...
pNp/h ($n = 3$) while the knockout produced 6.54 ± 1.12 μM pPp/h ($n = 3$), again indicating no significant impact of anxC3.1 disruption on protein secretion.

These data indicate that anxC3.1 does not have a major role in viability, sporulation or protein secretion. However, we have recently identified two annexin genes.
anxC3.1 and anxC3.2, each in A. fumigatus and A. niger [4], the existence of an as yet uncharacterised anxC3.2 homolog in A. niger could be significant.

Alternatively, the anxC3.1 disruptant phenotype may be more subtle than was anticipated. The D. discoideum annexin is the nearest evolutionary relative [3] which has been functionally characterised. Disruption of the annexin gene in D. discoideum did not result in any change to the exocytosis process [29] and it was suggested that the D. discoideum annexin is involved in calcium homeostasis.

### 3.4. anxC3.1 expression is constitutive

The expression pattern of the anxC3.1 gene under various growth conditions was analysed by semi-quantitative RT-PCR. A 600 bp fragment of the anxC3.1 gene and a 300 bp β-tubulin gene fragment were amplified from RNA samples and the relative levels of the two messages were then compared.

The influence of traffic through the secretory pathway on the level of anxC3.1 expression was considered by comparing RNA extracted from hyphae secreting various levels of a major secretory product, glucoamylase, using maltodextrin as an inducer and xylose and glucose as repressors of glucoamylase [30]. anxC3.1 levels did not alter with increased levels of traffic through the secretory pathway (Fig. 3). These data also indicate that the annexin expression is not regulated directly by carbon source, consistent with the absence of regulatory motifs such as the CREA (carbon catabolite repressor) binding motif in the promoter region of the gene [31,32].

The presence of nitrogen-regulation global transcription factor, AREA in the 5′ untranslated region of the annexin gene, at −446 and −470 relative to the start ATG, with the consensus (A/T)GATA(A/G), suggested nitrogen regulation of annexin expression. However, these are unlikely to be functional as the levels of anxC3.1 expression in cells grown with peptone, ammonium sulphate or ammonium nitrate as a sole nitrogen source did not show any significant variation (data not shown).

The effect of heat shock on anxC3.1 expression was determined by transferring mycelia grown to
mid-exponential phase at 30–42 °C for 1 and 2 h. Comparison of gene expression levels after 1 h heat shock showed no significant difference (data not shown).

We assessed *anxC3.1* gene expression in cultures of the parental *A. niger* strain, grown with altered levels of calcium within the growth media. Treatment of cultures with the calcium chelator (BAPTA) slowed mycelial growth (Fig. 4(a)) and also produced aberrant phenotypic effects such as shortening and thickening of hyphae, hyperbranching and ballooning of tips (Figs. 4(b) and (c)), a phenotype that has been reported to be induced by low calcium concentrations in other fungi [18,33–35]. Despite these severe morphological effects, expression of *anxC3.1* in these cultures and in a culture inoculated from the first BAPTA treated flask showed no significant difference in the level of expression compared to untreated controls (Fig. 4(d)).

In conclusion, we have carried out the first functional characterisation of a fungal annexin. We have shown that deletion of the gene has no effect on growth morphology or protein secretion, and that the gene is constitutively expressed. Interestingly, a similar situation is seen in mammals for annexin VII with which *anxc3.1* shares a number of similarities in sequence. Annexin
VII is considered to be a housekeeping gene as it does not show a typical TATA box, is expressed in a wide range of tissues and it is not assigned to any specific inducing stimuli [36].

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