Analysis of a homologue of the adducin head gene which is a potential target for the Dictyostelium STAT protein Dd-STATa

RYOTA AOSHIMA#, RIEKO HIRAOKA#, NAO SHIMADA# and TAKEFUMI KAWATA*
Department of Biology, Faculty of Science, Toho University, Funabashi, Chiba, Japan

ABSTRACT A Dd-STATa-null mutant, which is defective in expression of a Dictyostelium homologue of the metazoan STAT (signal transducers and activators of transcription) proteins, fails to culminate and this phenotype correlates with the loss of expression of various prestalk (pst) genes. An EST clone, SSK395, encodes a close homologue of the adducin amino-terminal head domain and harbors a putative actin-binding domain. We fused promoter fragments of the cognate gene, ahhA (adducin head homologue A), to a lacZ reporter and determined their expression pattern. The proximal promoter region is necessary for the expression of ahhA at an early (pre-aggregative) stage of development and this expression is Dd-STATa independent. The distal promoter region is necessary for expression at later stages of development in pstA cells, of the slug and in upper cup and pstAB cells during culmination. The distal region is partly Dd-STATa-dependent. The ahhA-null mutant develops almost normally until culmination, but it forms slanting culminants that tend to collapse on to the substratum. The mutant also occasionally forms fruiting bodies with swollen papillae and with constrictions in the prestalk region. The AhhA protein localizes to the stalk tube entrance and also to the upper cup cells and in cells at or near to the constricted region where an F-actin ring is localized. These findings suggest that Dd-STATa regulates culmination and may be necessary for straight downward elongation of the stalk, via the putative actin-binding protein AhhA.

KEY WORDS: transcription factor, adducin, actin, cell differentiation, Dictyostelium

Introduction

Organized movement of various types of cells is necessary for the development of multicellular organisms (Hou et al., 2002; Baum, 2004). Such cell movements involve modulations of the cytoskeleton, such as the formation of actin stress fibers (Xia and Karin, 2004; Leptin, 2005). In order to understand the mechanism underlying such organized cell movement, the cellular slime mould Dictyostelium serves as an excellent model. Dictyostelium cells aggregate by a chemotactic movement in response to cAMP to form a multicellular structure and the structure requires morphogenetic cell movement to complete development (Kimmel and Firtel, 2004).

The transcription factor Dd-STATa, a functional homologue of metazoan STAT (Signal Transducers and Activators of Transcription) proteins (Kawata et al., 1997), is necessary for efficient chemotactic cell movement at the aggregation stage and morphogenesis after the slug stage (Mohanty et al., 1999); suggesting that Dd-STATa may play a role in aggregation and multicellular development by regulating cell movement. The anterior prestalk region of the slug is composed of at least three subtypes of prestalk (pst) cells, pstA, pstO and pstAB cells. This is based on promoter analyses of two genes encoding extracellular matrix proteins, ecmA and ecmB (Jermyn et al., 1989; Gaskell et al., 1992). PstAB cells express both the ecmA and ecmB genes (Gaskell et al., 1992) and are localized in a funnel-shaped core of cells. The pstA cells express ecmA but not ecmB and ecmB expression is actively repressed in the pstA cells (Ceccarelli et al., 1991; Harwood et al., 1993). The repression of ecmB expression requires Dd-STATa; disruption of the Dd-STATa gene causes the pstA and pstO cells to express ecmB (Mohanty et al., 1999). There is also a failure to culminate in the

Abbreviations used in this paper: ahh, adducin head homologue; Dd, Dictyostelium discoideum; EST, expressed sequence tag; pst, prestalk; STAT, signal transducers and activators of transcription.
Dd-STATa null strain.

To further understand the molecular mechanism whereby culmination is regulated by Dd-STATa, we previously searched for Dd-STATa target genes that are expressed in the prestalk cells. These were analysed by microarrays and in situ hybridization (Maeda et al., 2003) and we identified 13 candidate genes (Shimada et al., 2004a). We previously analysed one of those genes, the SSK395(ahhA) gene. Here we show that ahhA encodes a protein similar to adducin, an actin-binding protein. We identify promoter regions that regulate its expression, investigate protein localization in the culminant and discuss its function.

Results

Prestalk cell-specific expression of the SSK395 gene

In a previous study, we identified 13 prestalk-specific genes whose expression is down regulated in the Dd-STATa null mutant (Shimada et al., 2004a). One of those is an EST clone SSK395 which is detectable at the tipped finger stage by in situ hybridization (Shimada et al., 2004a). We focused on the function of the gene encoding SSK395, because the expression pattern dramatically changes as development proceeds. At the first finger stage, expression in prespore cells became almost undetectable although weak expression in prestalk cells is still seen. The expression level becomes elevated again at the slug stage and it is observed throughout the entire prestalk region. Once culmination begins, expression in pstA cells disappears and localisation of the transcript then gradually shifts to the upper cup cells and the stalk tube entrance in mid culminants (Shimada et al., 2004a).

Semi-quantitative RT-PCR was used to monitor the expression time course of the SSK395 gene in Ax2 cells (Fig. 1A). In accordance with previous microarray observations (Iranfar et al., 2003), there are peaks of expression at 0 hour and 6–9 hours of development. SSK395 gene expression is maintained at later stages of development (Fig. 1A).

Reduced SSK395 gene expression in Dd-STATa null mutant at the late slug stage but not at the early stages

Interestingly, although expression at the tipped finger stage is detectable in the Dd-STATa null mutant, the transcript is hardly detectable in the mutant at the late slug stage (Shimada et al., 2004a). Therefore, only later expression appears to be positively regulated by Dd-STATa. To confirm whether expression of the SSK395 gene is Dd-STATa dependent, we performed semi-quantitative RT-PCR in a Dd-STATa null strain and compared expression to that in the parental strain, Ax2. SSK395 transcripts were detectable in both strains at the tipped finger stage (9 hours of development for Ax2 and 12 hours for the Dd-STATa null strain) (Fig. 1B). Interestingly, the transcript level at the mound stage in the mutant (8 hours of development) was rather stronger than that at the tipped finger stage. The expression level dropped during late first finger and early slug stages in both strains (12–15 hours of development for Ax2 and 16 hours for Dd-STATa null strain). Then, the expression increased slightly as development proceeded to the migratory slug stage in the Dd-STATa null strain (20 hours of development).

The SSK395 gene encodes a protein related to adducin

The SSK395 gene contains an open reading frame (ORF), which is capable of encoding a protein of 268 amino acids with a predicted molecular mass of 30.1 kDa. A database search using BLAST showed that the SSK395 gene encodes a protein with high homology to mammalian adducin (Fig. 2A). However, the gene does not encode the whole of adducin, it only encodes a domain corresponding to the head domain (Fig. 2B). Therefore, we named the SSK395 gene ahhA (adducin head-homologue A).

Promoter fragment of the ahhA gene confers prestalk-specific expression

Comparison of ahhA gene expression between Ax2 and Dd-STATa null strains by in situ hybridisation (Shimada et al., 2004a) and semi-quantitative RT-PCR (Fig. 1) imply the presence of at least two regulatory promoter elements; one is for the early stages and is independent of Dd-STATa and the other is for the later stages and is partly Dd-STATa dependent. To clarify whether this is true for ahhA gene expression, we amplified a promoter fragment of the ahhA gene and subcloned it into pDd-gal17(H+).
Adducin-like gene in Dictyostelium

**A**

Adducin-like gene in Dictyostelium

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhhA</td>
<td>C91531</td>
</tr>
</tbody>
</table>

**B**

**Fig. 2. Homology of the ahhA gene product with α-adducin, together with a scheme of the adducin protein and alignment with a putative actin-binding domain.**

(A) Alignment of the AhhA amino acid sequence with human α-adducin 1 (accession number L29296.1; Lin et al., 1995). Amino acid sequences of AhhA and human α-adducin 1 were aligned by using ClustalW program. Asterisks indicate identical amino acid residues between human α-adducin 1 and AhhA. Similarly, colons denote the amino acid residues with conservative change; dots indicate the residues with semi-conservative change. Amino acid position numbers are shown at both ends. Only the N-terminal portion corresponding to 420 residues is shown for human α-adducin 1.

(B) Schematic representation of adducin protein and an alignment of a putative actin-binding domain. The schematic structure of the α- or β-adducin subunit is shown at the top (adapted from Hughes and Bennett, 1995; Li et al., 1998). Adducin consists of head, neck and C-terminal tail regions. AhhA spans a region corresponding to head region of adducin. A sequence alignment of a putative actin-binding domain with conserved 27 amino acid-domain of already known actin-binding proteins and AhhA is shown at the bottom. Sequences of human α-adducin, human α-actinin (accession number P12814), human β-adducin (accession number P35612), Dictyostelium AhhA (accession number C91531), human β-spectrin (accession number P11277), human Dystrophin (accession number P11532), human endothelial ABP-280 (accession number P21333) and chicken fimbrin (accession number P19179) were aligned according to MultAlin program (Corpet 1998; http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl). Conserved amino acids are shaded. Hyphens indicate gaps of sequence alignment. For symbols of consensus sequence, ! is either I or V, $ is either L or M, % is either F or Y and # is either N,D,Q,E,B or Z. Amino acid position numbers are shown at both ends.
and the promoter activity was assayed by its β-galactosidase activity.

A DNA fragment, which has a 5' end-point 1081 bp upstream of the putative translational start codon of the \textit{ahhA} gene (\textit{ahhA}-5'\textasciitilde 1081::lacZ), gave a similar staining pattern to that observed in the \textit{in situ} hybridisation experiment (Shimada et al., 2004a). The signal was detected during aggregation (Fig. 4Aa). Later, a signal became detectable in the pstA region at the tipped mound and slug stages (Fig. 4Ab,c,d). At the Mexican hat stage, cells expressing lacZ are found in the tip region (Fig. 4Ae). The stained cells eventually locate as a thin ring-shaped structure in the prestalk region, stalk tube entrance and disk cells (Fig. 4Af,g).

Unlike the \textit{in situ} hybridisation results (Shimada et al., 2004a), the signal was also detected in the \textit{Dd-STATa}-null mutant transformed with \textit{ahhA}-5'\textasciitilde 1081::lacZ construct throughout entire development (Fig. 4B). This may reflect traces of β-galactosidase activity during the early stages of development because of its stable character. Therefore, we replaced the lacZ gene with ilc::lacZ, a fusion gene that produces unstable β-galactosidase (Detterbeck et al., 1994) and compared the staining pattern between in Ax2 and \textit{Dd-STATa}-null strains (Fig. 5).

The construct showed weakened staining, particularly in pstA cells, at late stages of development in the \textit{Dd-STATa}-null strain compared to the staining of Ax2 strain (Fig. 5B), implying the involvement of Dd-STATa in the expression of the \textit{ahhA} gene at late stages of development but not at early stages as already seen in the \textit{in situ} and RT-PCR experiments (Shimada et al., 2004a, Fig. 1). There is still a discrepancy between RT-PCR data and unstable lacZ data because of the absence of expression of the unstable β-galactosidase before tipped aggregates (Figs. 1 and 5). If a half-life of unstable β-galactosidase is the same, it could be possible that the stability of \textit{ahhA} mRNA may change depend on developmental time course, although the precise reason is unknown.

\textbf{Mapping of the \textit{ahhA} promoter regions necessary for early and late stage-specific expression}

To identify the regions necessary for expression at early and late stages, a series of 5'-3' deletion constructs were created. Deletion to 712 bp upstream of the putative translational start codon (termed ATG hereafter) weakened the staining in all pst cells at the slug and culminant stages in Ax2 and at the slug stage in the \textit{Dd-STATa}-null mutant (data not shown). Further deletion to 473 bp upstream of the ATG caused ectopic expression of the lacZ gene in the late prespore/spore region of the culminants in addition to the prestalk staining of slugs (Fig. 6A). This indicates that a late prespore/spore-specific repressor region is located between nt (nucleotide) -712 and nt -474.

Cells transformed with construct \textit{ahhA}-5'\textasciitilde 174::lacZ showed no pst- nor late staining. The observed diffuse staining (not a specific pst-staining) may due to scattered cells expressing stable β-galactosidase from beginning of development or pre-aggregation stage (Fig. 6B). Cells transformed with construct \textit{ahhA}-5'\textasciitilde 132::lacZ lacked staining entirely at all stages of development (Fig. 6C). These results demonstrate that the region necessary for expression at the later stages in pstA cells is between nt -473 and -175, while expression at the early stages requires sequences between nt -174 and -132.

\textbf{Disruption of the \textit{ahhA} gene leads to a tendency to form slanting culminants and aberrant papillae}

To determine the function of the AhhA protein, mutants lacking
the \textit{ahhA} gene were created by homologous recombination (Fig. 7A). Two independent targeted clones were isolated by PCR screening using genomic DNA as a template and a semi-quantitative RT-PCR analysis showed that the \textit{ahhA} transcript is almost undetectable (Fig. 7A). When the \textit{ahhA} cells were allowed to develop on either phosphate-buffered filters or on water agar plates, the \textit{ahhA} cells developed more rapidly, by approximately one hour (data not shown), but morphologically normally until early culmination (data not shown). At the mid stage of culmination, ~5% of the mutants formed swollen papillae with a constriction at the prespore-prestalk boundary (Fig. 7B c,d). The terminal structure of most mutant entities was indistinguishable from that of the parental strain, Ax2, except that the mutant formed a slanting fruiting body and easily collapsed on to the substratum (Fig. 7B e). This latter, weaker phenotype is more prevalent, as more than 50% of the culminants show such behaviour (data not shown). All these phenotypic defects were rescued by expressing AhhA protein under the control of its own promoter (Fig. 7Bf). Therefore, all defects of the mutant are attributed to inactivation of the \textit{ahhA} gene.

Since several null mutants of genes encoding actin-binding protein show a more severe phenotype when they are allowed to develop on soil (Ponte \textit{et al.}, 2000), we tested the phenotype \textit{ahhA}-cells on soil plates. However, no phenotypic change was observed in such experiments (data not shown).

\section*{Localization of the AhhA protein at the tip, pstO cells and cells near the constricted region}

In order to elucidate AhhA function, we made a construct, which expresses AhhA protein with a human c-myc epitope tag, under the control of the \textit{ahhA} promoter (Fig. 8A). The construct was transformed into the \textit{ahhA}-null mutant and AhhA protein...
expression was monitored. Western blotting of total cell proteins from three independent clones identified a protein with an approximate molecular weight of 30 kDa, which matched well to the size predicted from the AhhA amino acid sequence (Fig. 8B).

Next we examined AhhA protein localization by immunohistochemical staining (Fig. 8C). The protein localized at the stalk tube entrance and pstO cells (arrowhead). The protein also localized in cells near the constricted region, where the ahha transcript localized (Shimada et al., 2004a; Fig. 4A). Since the AhhA has a putative actin-binding domain and the F-actin ring localizes at the constricted region, we tried co-staining with fluorescent phalloidin. As shown in Fig. 8C, AhhA protein co-localized with F-actin at the cells near the constriction (coloured arrows).

**Discussion**

**A new Dd-STATa-dependent marker expressed in prestalk cells**

In this study we identify a new marker gene, ahha, which is expressed in all cells at the early stages and comes to be expressed in prestalk cells at later developmental stages, after the tipped finger stage (Fig. 4A). Expression in pstA cells at stages later than the slug stage is down-regulated in the Dd-STATa-null mutant, while expression up to the tipped finger stages is not (Shimada et al., 2004a; Figs. 1 and 5). The spatial and temporal patterns of ahha expression look almost the same in Ax2 and Dd-STATa-null cells but longer staining is required to visualize it (Figs. 1 and 4). However, lacZ staining in pstA cells is down-regulated in the Dd-STATa-null mutant compared to the staining in pstO cells (see Fig. 5d). The prestalk marker gene ecmA shows a similar expression pattern at the slug stage but there is a critical difference in the expression patterns of these two genes during culmination. During culmination, the ecmA gene is expressed in all prestalk cells. However, expression of the ahha gene in the pstA cells disappears and then re-appears in the upper cup cells, pstAB cells, stalk tube entrance and disk cells. There is no detectable expression in the bottom part of the stalk (Fig. 4A). This expression pattern is rather similar to the ecmB gene expression pattern. However, there is a difference in the expression pattern in the late culminant. Unlike the ahha gene, the ecmB gene is expressed in the entire stalk. Most notably, the ecmB gene is also expressed in the lower cup cells. Therefore, the ahha gene is a novel type of prestalk marker gene whose expression is partly dependent on Dd-STATa. It provides a tool to investigate the function of Dd-STATa.

**Subregions of the ahha promoter directing early and late stage expression**

Although the ahha gene is expressed throughout development, the transcript amount and spatial expression pattern of ahha show very complicated profiles (Shimada et al., 2004a; Figs. 1A, 4A). Deletion analysis of the ahha promoter mapped a region necessary for expression in prestalk cells (particularly in pstA cells) at “later stages” to between -473 and -175, a region necessary for expression at early stages to between -174 and -133 (Figs. 3–6). A region between -712 and -473 must have a negative regulatory element(s) repressing ahha expression in late prespore/spore cells and a region between -473 and -175 may contain a region required for the expression in those cells (Fig. 6A). The expression pattern of the ahha-5’-473::lacZ deletion construct in those cells is similar to that observed for spiA gene, a marker gene of late prespore/spore cells (Richardson et al., 1994).

Since 5’-deletion constructs for further fine mapping are all unstable in bacterial cells, we have been unable to identify sequences necessary for prestalk cell expression at later stages in the region between -473 and -175 (data not shown). There are several GC sequences in this region (Fig. 3). Placing such GC sequences upstream of the ahha-5’-473::lacZ deletion construct gave no staining (data not shown). Therefore, it is likely that at least two GC sequences in this region are necessary for prestalk specific expression and the sequence element...
Fig. 7. Knock out mutants of ahhA gene. (A) Creation of ahhA knock out construct and screening of ahhA strains. Knock out construct of ahhA gene. BsrR cassette was inserted as described in Materials and Methods at 81 bp down stream from the ahhA gene translational start site (panel a). Targeted gene disruption was screened by genomic PCR with primer pair, Bsr-1 and 396-ORF-2 (data not shown). The disruption was further confirmed by both genomic PCR (data not shown) and RT-PCR (panel b). Genomic PCR was performed with primer pair 395-G2 and 395-G6 for two independently isolated ahhA-null mutants (KO1 and KO2). One microgram of total RNAs isolated from wild-type (Ax2) and the ahhA-null mutant (KO1) at vegetative growing stage were used for RT-PCR. Ribosomal RNAs (1 µg total RNA/lane) were electrophoresed and stained with EtBr for loading standard. (B) Developmental phenotype of ahhA-null mutant. Cells of wild-type (Ax2, panels a and b), the ahhA-null mutant (panels c-e) and the rescued strain (panel f) were allowed to develop on phosphate-buffered filters to mid-culmination stage (panels a, c, d and f). Panels b and e show the overhead views of the late-culminants of the wild-type (Ax2) and the ahhA-null cells, respectively. Arrows indicate the swollen papillae of the mutant (see text for details).

Fig. 8. Detection of the AhhA protein in the culminants. (A) Expression construct of the AhhA protein, pDd-ahhA-myc. The ORF region of the ahhA gene was placed under the control of its own promoter. Human c-myc epitope tag was introduced into the unique HindIII site in the coding region. (B) Expression of the AhhA protein as detected by Western blotting. pDd-ahhA-myc was transformed into ahhA-null cells and three independent transformants (r1, r2, r3) were analysed. For Western analysis, approximately 20 µg of total cell protein was loaded per each lane and analysed as described in Materials and Methods. Lane 1 indicates Ax2 proteins, lanes 2-4 correspond to the transformants r1-r3, respectively. A protein band with a molecular weight of approximately 30 kDa (arrowhead) was visible in lanes of transformants but not of Ax2, thus, supposed to be specific AhhA band. (C) Spatial expression of the AhhA protein as detected by immunohistochemical staining. Photographs (a) and (c) are relief contrast images, (b,d,e,f) are fluorescent images of the mid-culminants of Ax2 strain (a,b) and AhhA::c-myc expressing strain (c-f). (b,d) are images of anti-c-myc antibody detected by Alexa Fluor 488 phallolidin (Invitrogen). Coloured arrows indicate localization of AhhA protein near the constricted region of late-culminants; the arrowhead indicates pso localization of the AhhA protein (see text for details).
responsible for expression in prestalk cells remains to be identified.

**Putative function of the AhhA protein**

AhhA is a protein with a putative actin-binding domain and it is highly homologous to mammalian adducin (Fig. 2). Data base search shows that there is no other gene encoding an adducin-like protein in the *Dictyostelium* genome (Eichinger et al., 2005). Adducin is a heterodimeric cytoskeleton protein involved with actin and spectrin in forming specialized contact sites in various multicellular organisms (Yue and Spradling, 1992; Robinson et al., 1994). Interestingly, the AhhA protein lacks domains corresponding to neck and protease-sensitive carboxyl-terminal tail but harbors a globular head domain (Hughes and Bennett, 1995). The C-terminal tail is a target of protein kinase C and Rho kinase and has an important role in forming a complex with fast growing ends of actin filaments that recruit spectrin (Hughes and Bennet, 1995; Li et al., 1998). Therefore, the AhhA protein may not be an authentic adducin.

Despite lacking the C-terminal domain of adducin, localization of AhhA protein is in cells where F-actin is enriched and where there is a constriction in the stalk tube (Grimson et al., 2000; Fig. 8C). Although there is no evidence to show a direct interaction of AhhA protein with F-actin, the above result and the presence of putative actin-binding domain imply that the AhhA protein may interact with F-actin.

Inactivation of the *ahhA* gene causes almost no phenotypic change but *ahhA*-null cells form aberrant culminants; slanting culminants, frequently than is observed for wild-type cells (Fig. 7C). The mutants easily collapse on to the substratum (Fig. 7Be). Under microscopic observation the mutant stalk is indistinguishable from that of wild-type stalk (data not shown), therefore, the collapse may be caused by the slanting of culminants but not by the weakness of the stalk. In addition, the AhhA protein co-localizes at a region near or where the stalk tube is constricted. This may indicate that AhhA is necessary for the correct downward elongation of the stalk. It may control the shape of the culminant through interaction with other molecules, possibly F-actin or adherens junction molecules (Grimson et al., 2000; Coates and Harwood, 2001; Coates, 2003; Williams and Harwood, 2003). A protein supposed to be adducin has been identified in *Physarum polycephalum*. It forms large aggregates of actin filaments and modifies actin-myosin interactions by blocking it (Ogihara and Tonomura, 1982). The AhhA protein may have similar function, although it remains to be elucidated.

A link between Dd-STATa signaling and the AhhA protein and the cytoskeleton?

Our finding that the *ahhA* is down-regulated in the *Dd-STATa*-null mutant at later stages of development (Figs. 1 and 5), suggests that Dd-STATa has a role(s) during culmination of regulating this adducin-like protein. As the protein harbors a putative actin-binding domain, this provides a tool to investigate Dd-STATa function on morphogenesis, through the cytoskeleton.

**Materials and Methods**

**Cells and growth conditions**

*D. discoideum* Ax2 and *Dd-STATa*-null cells were cultured axenically in HL5 medium at 22°C. Cells of *Dd-STATa*-null and *SSK395(ahhA)*-null strains were grown in HL5 supplemented with 10 µg/ml blasticidin S (Kaken Pharmaceutical, Tokyo, Japan). Transformants containing the Neo<sup>+</sup> cassette were selected in HL5 supplemented with 10 µg/ml G418 (geneticin; ICN Biochemicals Inc., Ohio, USA) and then the concentration of G418 was increased to 20–80 µg/ml.

**Genomic DNA cloning**

The genomic sequence of the *SSK395(ahhA)* gene was obtained via a BLAST search (http://dictybase.org/db/cgi-bin/blast.pl) using the SSK395 cDNA sequence from the Dicty-cDNA database (http://www.csm.bio.tsukuba.ac.jp/cDNAproject.html) as a search term. The genomic fragment was amplified by PCR using the oligonucleotides 395-G2 (5'-TGC TCT AGA GCA CAT TAG TTT TAA CTC ATT TAG CTC-3') and 395-G6 (5'-CCG CTC GAG CGG CTT TGG TGA AAC AAG CAG CTG CTG-3') as primers (see Fig. 7A) and genomic DNA from the Ax2 strain as the template. A PCR fragment of approximately 1.7 kbp, was amplified and subsequently subcloned into pCR4-TOPO (Invitrogen Life Technologies) to yield pTOPO-395.

**Construction of targeting vector**

An *SSK395(ahhA)* targeting vector was constructed by use of the inverse PCR. Both oligonucleotides 395-G1 (5’-CGC AAT TCA TGA TTT CAA AGG ATT AAC TAA TGA AAG GTC ATC ACT ACT TG-3') and 395-ORF-2 (5’-CGG CTC GAG TTA ATT TTT AAA AGA ATC ATC TAA TTT C-3') were selected in 96-wells plates containing HL5 medium supplemented with 10 µg/ml blasticidin S. To screen the clones for those in which the *SSK395(ahhA)* gene had been successfully targeted, genomic DNA from each clone was amplified by PCR using two pairs of primers 395-G5 (5’-CGC AAT TCA TGA TTT CAA AGG ATT AAC TAA TGA AAG GTC ATC ACT ACT TG-3') and 395-G2 (5’-CCG CTC GAG CGG CTT TGG TGA AAC AAG CAG CTG CTG-3') with 10 µM primer and 100 µM dNTP. PCR were performed for 35 cycles. The PCR products were purified and ligated with a bsr<sup>+</sup> cassette digested with BanHI. The targeting vector was digested with BanHI and ligated with a bsr<sup>+</sup> cassette digested with BanHI.

**Screening of the SSK395(ahhA)-null strains**

The target vector was digested with *NotI* and *SpeI*. After phenol/chloroform extraction followed by ethanol precipitation of the vector DNA, Ax2 cells were transformed by electroporation. Transformants were selected in 96-wells plates containing HL5 medium supplemented with 10 µg/ml blasticidin S. To screen the clones for those in which the *SSK395(ahhA)* gene had been successfully targeted, genomic DNA from each clone was amplified by PCR using two pairs of primers 395-ORF-2 (5’-CCG CTC GAG CGG CTT TGG TGA AAC AAG CAG CTG CTG-3’) and 395-G2 / 395-ORF-2 (see Fig. 7A).

**Analysis of gene expression by semi-quantitative RT-PCR**

RT-PCR was performed as described previously (Shimada et al., 2004b) by RNA LA PCR Kit (AMV) version 1.1 (TaKaRa, Kyoto, Japan) using a pair of primers 395-ORF-1 (5’-CGG AAT TCA TGA TTT CAA AGG ATT AAC TAA TGA AAG GTC ATC ACT ACT TG-3’) and 395-G5 (5’-CGC AAT TCA TGA TTT CAA AGG ATT AAC TAA TGA AAG GTC ATC ACT ACT TG-3’) for *Dd-STATa* gene. The PCR products of *Dd-STATa* were also amplified by PCR using two pairs of primers 395-G5 (5’-CGC AAT TCA TGA TTT CAA AGG ATT AAC TAA TGA AAG GTC ATC ACT ACT TG-3’) and 395-G2 (5’-CCG CTC GAG CGG CTT TGG TGA AAC AAG CAG CTG CTG-3’).

**Construction of various lacZ fusion construct and expression construct**

The promoter of the *SSK395* gene was amplified by PCR to add an *XbaI* site at its 5’ end and a *BglII* site at its 3’ end. After digestion with *XbaI* and *BglII*, the fragment was gel-purified and subcloned into *XbaI*/*BglII* cut pDd-gal17(H+*) (Harwood and Drury, 1990) to make pSSK395::lacZ<sup>+</sup>. For 5’ deletions, primers corresponding to each deletion point with *XbaI* site at the 5’ end were used to amplify the deleted fragment, then subcloned into pDd-gal17(H+*) as above.

Construction of human c-myc-tagged SSK395(ahhA) expression vector, pDd-gal17H(+*) was digested with *BglII* and *XhoI* to delete the lacZ gene to produce pDd-gal17H(+*)∆gal. The genomic fragment was amplified by PCR with a pair of oligonucleotides 395-G2 and 395-ORF-2 as primers using genomic DNA from Ax2 cells as a template. A DNA fragment, which contains an entire *ahhA* ORF and its promoter was amplified and subcloned into pCR2.1-TOPO (Invitrogen Life Technologies) to yield pTOPO-395.

For construction of human c-myc-tagged SSK395(ahhA) expression vector, pDd-gal17H(+*) was digested with *BglII* and *XhoI* to delete the lacZ gene to produce pDd-gal17H(+*)∆gal. The genomic fragment was amplified by PCR with a pair of oligonucleotides 395-G2 and 395-ORF-2 as primers using genomic DNA from Ax2 cells as a template. A DNA fragment, which contains an entire *ahhA* ORF and its promoter was amplified and subcloned into pCR2.1-TOPO (Invitrogen Life Technologies) to yield pTOPO-395.
and its promoter was gel purified and ligated into E:: pb site of pDd-gal17H(+)/gal to yield pDd-ahhA. As a human c-myc epitope EQKLISeEDL, two oligonucleotides corresponding to sense (5‘-AGCT GAA AAA ATT TCT GAA GAT CTT GC-3‘) and antisense (5‘-AGCT GC AAG ATC TTC TCT AGA AAT AAG TTG TTC-3‘) strands were annealed then ligated into an unique HindIII site of pDd-ahhA to make pDd-ahhA-myc (see Fig. 8). The HindIII site was located 584 bp downstream from translational start codon of the SSK395(ahhA) ORF. Direction of the inserted double stranded oligonucleotides and the reading frame was confirmed by PCR and sequencing.

**Promoter analysis by β-galactosidase staining**

For the detection of promoter activity, cells transformed with various deleted promoter fragments fused to lacZ (Dettetbeek et al., 1994) reporter plasmids were grown and developed on nitrocellulose filters. Expression and staining were performed as described (Dingermann et al., 1989).

**Western analysis and immunohistochemical staining**

For Western analysis, cells were solubilized in SDS sample buffer containing EDTA-free Complete Mini (Roche, Penzberg, Germany) as proteinase inhibitors. The proteins were analyzed on 5–20% gradient SDS-polyacrylamide gels and blotted onto Hybond-ECL filters (Amersham Biosciences, Ltd. UK). The filters were blocked and detected by Promega Peroxidase II AP System with Stabilized Substrate (Amersham Biosciences, Ltd, UK). The filters were blocked and de-

For Western analysis, cells were solubilized in SDS sample buffer containing EDTA-free Complete Mini (Roche, Penzberg, Germany) as proteinase inhibitors. The proteins were analyzed on 5–20% gradient SDS-polyacrylamide gels and blotted onto Hybond-ECL filters (Amersham Biosciences, Ltd. UK). The filters were blocked and detected by Promega Peroxidase II AP System with Stabilized Substrate (Amersham Biosciences, Ltd, UK). The filters were blocked and de-

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (JST) to T. Kawata (#17570190) and a Grant-in-Aid for JSPS Fellowships for Young Scientists to N. Shimada (#17.2751). N.S. is a JSTS Research Fellow. We are grateful to Professor Jeffrey G. Williams, University of Dundee, UK, for his critical reading of the manuscript and encouraging comments on the entire work.

**References**


...ated cell signaling in a non-metazoan organism. Nature 408:727-731.


...activité des isopéptides dans le cytoplasme de Dictyostelium discoideum. Dev. Genet. 5:303-310.


OGIHARA, S. and TONOMURA, Y. (1982) A novel 36,000-dalton actin-binding protein purified from microfilaments in Physarum plasmodium which aggre-


Received: October 2005
Reviewed by Referees: December 2005
Modified by Authors and Accepted for Publication: February 2006
Published Online: May 2006