BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 75–78 (1997)
ARTICLE NO. RC976907

A Novel Fungal Gene Encoding Chitin Synthase with a Myosin Motor-like Domain

Makoto Fujiwara,[1] Hiroyuki Horiuchi, Akinori Ohta, and Masamichi Takagi[2]
Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

Received May 29, 1997

A *csmA* gene that encodes chitin synthase with a myosin motor-like domain was isolated from the filamentous fungus *Aspergillus nidulans*. Initially, we obtained the *csmA* as a homolog of the *Aspergillus fumigatus chsE*-partial fragment. A large open reading frame encoding a polypeptide of 1,852 a.a. was identified by determining the cDNA sequences. The chitin synthase conserved region was situated at the C-terminus and classified into class V as reported previously. On the other hand, the N-terminal region showed significant similarity to myosin motors and could not be classified into any types of myosins identified so far. Thus, it is suggested that this is the first report of unconventional myosin fused to a metabolic enzyme. The finding of this new type of chitin synthase gene suggests that localization of chitin synthesis may be guided by association with cytoskeletal structures.

Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine, is one of the major structural components of the fungal cell wall (1). Chitin synthases are the membrane-bound proteins responsible for the catalytic polymerization of *N*-acetylglucosamine from UDP-sugar (2). The enzymatic aspects and physiological functions of chitin synthases have been well characterized in the budding yeast *Saccharomyces cerevisiae*, in which chitin is a minor component (2, 3). However, there is little information about chitin synthases in filamentous fungi.

Fungal chitin synthases are encoded by *chs* genes. Their gene products commonly possess a conserved C-terminal domain which contains putative transmembrane domains. The primary structures of many fungal chitin synthases have already been reported, and they have been divided into five groups - classes I through V - on the basis of their conserved region structures (4-6). Four chitin synthase genes have so far been cloned from an ascomycete, *Aspergillus nidulans* by our group, and named *chsA, chsB, chsC* and *chsD*, which correspond to classes II, III, I and IV, respectively (7-9).

In this report, we have described the isolation of a novel gene (*csmA*) from *A. nidulans*, and shown that *csmA* encodes an N-terminal myosin motor-like domain outside the chitin synthase-conserved domain, and that both domains possess peculiar structures compared with their related proteins.

MATERIALS AND METHODS

Amplification of the A. fumigatus chsE homolog from A. nidulans. Aspergillus nidulans total DNA was extracted as described previously (10). PCR was run with a degenerate primer set, AnE1 5′-TGGGGA-TCCCA(A/G)GTNTA(T/C)GA(A/G)TA(T/C)TA-3′ and AnE2 5′-ATA-GAATTCTTIATCCAIC(T/G)IC(T/G)IC(T/G)(T/C)TG-3′ (I, inosine; N, A/C/G/T), for *A. nidulans* total DNA from strain FGSC89 (Fungal Genetic Stock Center, U.S.A.). 40 cycles were run consisting of a 0.5-min melting step at 94°C, a 1-min annealing step at 50°C, and a 1.5-min extention step at 72°C.

Isolation of csmA. With the PCR fragment as a probe, positive phage clones were screened from the genomic DNA library in λ2001 (7) by plaque hybridization. Two positive phage clones, M1 and M3 were obtained. The 6.7-kb *Kpn*I-*Xho*I and 7.0-kb *Xba*I fragments from M1 and M3 were cloned into pUC119 at the sites of *Kpn*I-*Sal*I and the *Xba*I site to yield pMK10 and pM3X2, respectively.

Northern blot analysis. Total RNA from FGSC89 cultured on yeast extract-glucose medium (11) for 16.5-hr were was extracted as described previously (9). Poly(A)$^+$RNA were purified with mRNA purification Kit (Pharmacia Biotech). 2μg of poly(A)$^+$RNA were electrophoresed with size markers and blotted on Hybond-N+ membrane (Amersham). ^{32}P-labeled two DNA fragments of 1.0-kb *Spe*I-*Xho*I (probe1) and 2.3-kb *Hin*dIII-*Xba*I (probe2) from pM3X2 and pMK10, respectively, were used as probes. Hybridization and washing were carried out as suggested by the manufacturer. Signals were detected with BAS2000 Bioimage analyzer (Fuji Film).

RACE and primer extention analysis. RACE experiments were done using Marathon cDNA Amplification Kit (Clontech) and Expand High Fidelity PCR System (Boehringer Mannheim). Two oligonucleotide primers, R1-2 5′-GAAATCCCACAATGATGAATACGGC-3′ and R3-1 5′-GCATCATATTGCAGTTCCAGGAAC-3′ were used to syn-

[1] Present address: Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan.
[2] To whom correspondence should be addressed at Laboratory of Cellular Genetics, Department of Biotechnology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan. Fax: +81-3-3812-9246. E-mail: amtakag@hongo.ecc.u-tokyo.ac.jp.

0006-291X/97 $25.00

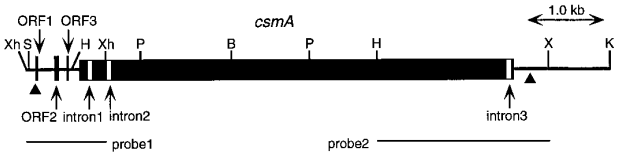

FIG. 1. Schematic representation of the genomic clone of *csmA*. The long ORF of *csmA* and three short ORFs (ORF1-ORF3) are shown. The positions of three introns are also shown. Abbreviations of representative restriction sites are as follows: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sp, *Spe*I; X, *Xba*I; Xh, *Xho*I. Two bars, probe1 and probe2, correspond to the DNA probes used for Northern blot analysis (see Fig. 2).

thesize the cDNA for 5′-RACE. R1-1 5′-GAACTTTTCACGCCATGCAATTTGG-3′, R2-1 5′-GTTTTACCGGCGCCACTCTCGC-3′, R2-3 5′-CGATGTGGAGGATAGCAGCCAAG-3′ and so on were used to amplify the 5′-cDNA ends. R1-3 5′-TTCATCATCTGTCAGGTTCCAGCC-3′, R1-4 5′-GCAGTACGACGATAAACGCAAGC-3′ and so on were used to amplify the 3′-cDNA ends. Primer extension analysis was done using R4-1 5′-CGATAGCGGAGAAGCCCGTAACTTGCTAATACA-3′ as a primer. MMLV-RT (Gibco BRL) was used for cDNA synthesis.

RESULTS

The *chsE* gene fragment among six chitin synthase related genes was obtained by PCR from an opportunistic pathogen, *Aspergillus fumigatus* (12). The predicted amino acid sequence of ChsE showed some similarity to the *S. cerevisiae* Chs3p, but could not be grouped into the known groups. This suggested that there could be another chitin synthase gene in *A. nidulans*. We

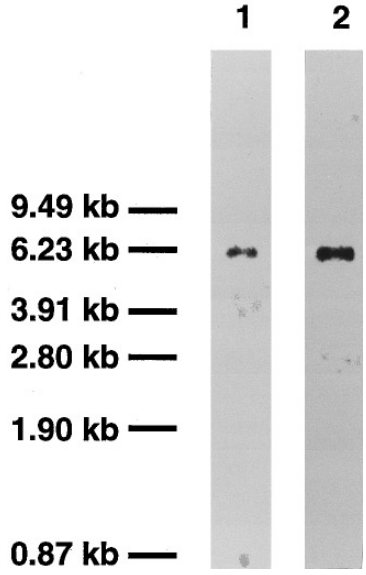

FIG. 2. Northern blot analysis. 2μg of poly(A)$^+$RNA (lanes 1 and 2) from mycelia of strain FGSC89 was electrophoresed with size markers (−kb, 9.49, 6.23, 3.91, 2.80, 1.90, 0.87) and probed with two DNA fragments, probe1 (lane 1) and probe2 (lane2), respectively (see Materials and Methods and Fig. 1).

```
   1 MVGTLPAGHTPSHVQSSLPSLPAHLQSDTHLTAHLASRFHVGLPTARLSSHALISLNNYT
  61 SSSKGPDGGKEGSAMGETEDLARRAYTRLGARGENQAIVFLGESGAGKTTLRSHLLSSFL
 121SFSSTPLSSKLSYAAFIFDTLTTTKSLTTPTASKAGLFLELQYDASSSVNPTLIGGKIID
 181HRLERSRIASVPTGERSFHVLYYLLAGTSAAEKAHLGLDSPIHVTTAGGRLSSADHKRWR
 241YLGHPTQLKVGINDADGFQHFKTALRKLEFPRSEIAEICQILAAILHIGQLDFGSGQATL
 301TGAEESGGYSHEGGETVTVVKNKDVLSIIAAFLGLGVGELEASFGYRTKTIHRERVTVML
 361DPKGARRSADELSRVLYSLLVAYVIENVNQRICAAEDSVANTVSIIDFPGFAQACSTGST
 421LDQLLNNAACESLYNFCLRSFFDRKADMLEREEVAVPATSYFDNTDAVRGLLKQGNGLLS
 481ILDDQTRRGRTDAQFVEAVRRRFENKNPAITAGASGSGNGYGMVSQNARSSFTVKHFAGE
 541VDYSATGLLEENGEVISGDLMNLMKSTRSDFVRELFGQEALQTVAHPKEKTAIMQAQVSS
 601KPLRMPSMARRKTSPASRLTFDATPAEDPYETESQTGSSAKNSSAKRKSGMLMGGMQCAA
 661GQFLSSLDIVNKCLTSGNLNPYFVFCLKPNDRRIANQFDSKCVRTQIQTLGIAEISQRLR
 721NADFSVFLPFAEFLGLAEVGNVVVGSDKEKSEVVLDEKRWPGNEARVGSTGVFLSERCWA
 781DLAKVGERVIPSFAAEDDGGDALLHPRTANYADSKVRLLNPSDHSPGAYIYGDESKQASN
 841TSRDFDGRSDAGYSAFNSGDMFHNLETREQMLEKGNEKQMEEVDEVPVSGSRKRWMAIVW
 901LLTFYIPTPAIRYIGRMKRKDIQIAWREKFAINLLIWLACAIAVFIIVGFPSLICPTQHV
 961YSPAELSSHDGKDGHSSYTSIRGLVLDLGEFMDSHYPGIVPDSALKKYAGVDSTALFPVQ
1021 VSALCLGKDGNVDPKVLLDYKPTNFSGSVTSTSSGDPNSVYHDFRYFRDDYRPDWYAEQM
1081 IYLRANYYKGWIGYSSEYLHTLASKSQNVASINGKIYDLTSYIAGGRRIQGREGDDTTGI
1141 DYDFMDSLVVDLFQQKAGEDITKYWEDLPLTPKLRVDMMDCLNNLFIVGHVDTRNSTQCQ
1201 FARYFILAISVLICSVIVFKFFAALQFGKKNVPENLDKFIICQVPAYTEDEESLRRAIDS
1261 MARMQYDDKRKLLVVICDGMIIGQGNDRPTPRIVLDILGVPESVDPEPLSFESLGEGMKQ
1321 HNMGKVYSGLYEVQGHIVPFLVVVKVGKPSEVSRPGNRGKRDSQMVLMRFLNRVHYNLPM
1381 SPMELEMHHHIRNIIGVNPTFYEFILQVDADTVVAPDAATRMVSSCLNDTRIIGVCGETS
1441 LTNAKTSAVTMIQVYEYYISHNLTKAFESLFGSITCLPGCFTMYRIRSAESGKPLFVSKE
1501 IVEAYSEIRVDTLHMKNLLHLGEDRYLTTLLLKHHPKFKTKYNFRAQAYTIAPESWTVFL
1561 SQRRRWINSTVHNLVELIPLQQLCGFCCFSMRFVVFIDLISTIIMPVTVAYIVYLIVWLV
1621 RDTSTIPWTSFLLLAAIYGLQAIIFIVRRKWEMIGWMIIYILAIPVYSLALPLYSFWHMD
1681 DFSWGNTRIITGEKGRKIVISDEGKFDPASIPKKRWEEYQAELWEAQTSRDDRSEISGIS
1741 YGTKYHPATQSEYGFPGSRPMSQLELPRHMSRMSLAPSEMMSRHMDMELEDVNLPSDDAI
1801 LSEIRDILRTADLMTVTKKNIKQELERRFGVNLDAKRPYINSATEAVLSGNL
```

FIG. 3. The predicted amino acid sequence of *csmA* product. Two boxes indicate the ATP-binding motif (P-loop) and the amplified region by PCR with AnE1 and AnE2, respectively. The genomic DNA and cDNA sequences of *csmA* will appear in the DDBJ databases under accession No. AB000125.

could obtain a single PCR fragment as a homolog of the *chsE*-PCR fragment. Then, we isolated and determined the homologous sequence of the genomic DNA fragment from the *A. nidulans* gene library. The sequence of cDNA was determined by 5′- and 3′-RACE methods and primer extension analysis (Fig. 1). A long ORF (5.5-kb) could be identified (Fig. 1). The transcript contained three short ORFs upstream of the main ORF, indicating that the gene expression could be regulated at the translational level. The approximate size (6.2-kb) of the transcript was confirmed by Northern blot analysis with two different probes (Fig. 2).

The amino acid sequence of the ORF comprises 1852 residues corresponding to a molecular mass of 206-kDa (Fig. 3). This chitin synthase gene encodes a fairly large molecule as compared with other chitin synthases (Fig. 4). The N-terminal domain of about 700 residues showed significant similarity to myosins, motor proteins that possess ATP-hydrolytic and actin binding activities to drive them along actin filaments (Fig. 4). Myosin is composed of an evolutionally conserved motor domain and a functionally variant C-terminal domain (13, 14). By phylogenetic analysis of the motor regions, myosins have been classified into eleven types (14). The N-terminal domain of the ORF showed simi-

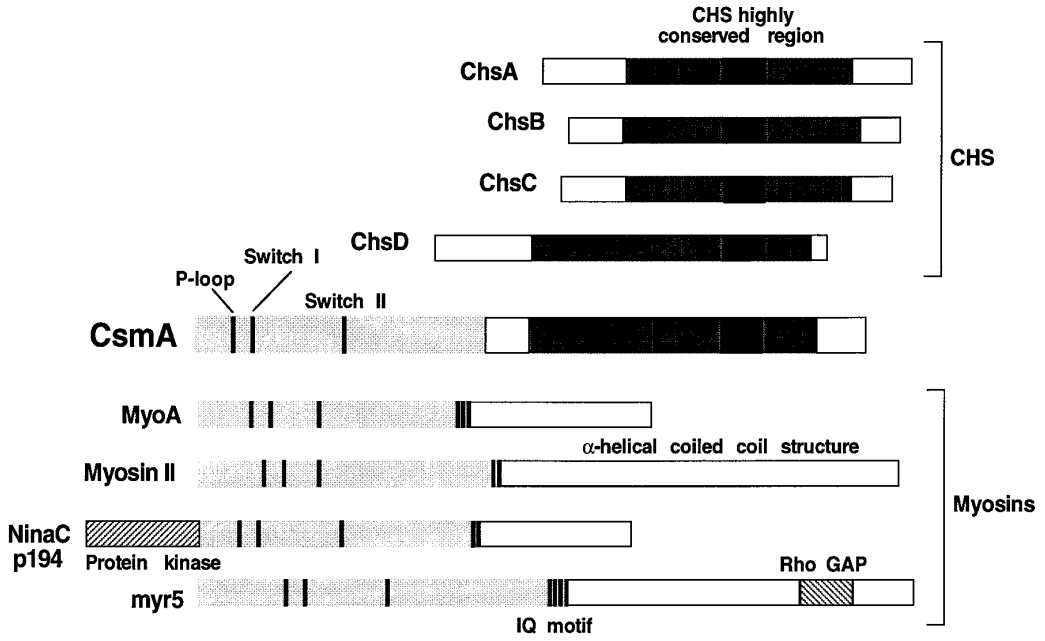

FIG. 4. Comparison of the structure of CsmA with other related proteins. ChsA, ChsB, ChsC, and ChsD, from *A. nidulans;* MyoA, from *A. nidulans;* Myosin II, Chicken muscle Myosin II; NinaC, from *Drosophila;* myr5 from pig. Open boxes and black boxes show non-conserved regions and highly conserved regions, respectively. Shaded boxes show slightly conserved regions.

larity to a broad range of myosins, e.g., myr1 (type-I, 22.5% identity in 382 a.a.), myosin I from chicken brush border (type-I, 22.4% in 353 a.a.), *Saccharomyces* Myo1p (type-II, 17.8% in 343 a.a.), *Drosophila* NinaC (type-III, 19.7% in 289 a.a.), pig myosin-VI (type-VI, 23.1% 553 a.a.) and *Arabidopsis* MYA2 (type-XI, 24.2% in 252 a.a.), but could not be classified into any known type of myosin family (data not shown). There was no potential IQ-motif or α-helical coiled-coil structure, which are present in unconventional or conventional myosins. In spite of the low similarity to myosin motors, the positions of the P-loop, the switch I and the switch II, and the essential residues in these loops are conserved (Fig. 4) (15). The N-terminal domain is at least likely to show the nucleotide hydrolytic activity that results in the conformational change. The features described above indicate that this is the first reported example of a myosin fused to a metabolic enzyme, although there are two known distinct uncoventional myosins, *Drosophila* NinaC and myr5, that possess a protein kinase domain and a Rho-GAP domain, respectively (16, 17). We designated this gene *csmA* (*c*hitin *s*ynthase with *m*yosin motor-like domain).

The C-terminal half of *csmA* product (CsmA) showed high similarity to class IV chitin synthases (data not shown). However, it could be grouped into a new class, class V, together with the *A. fumigatus* ChsE (data not shown). Its sequence was almost the same as that of the class V-chitin synthase from *A. nidulans* strain FGSC4 recently reported and named ChsD by Specht et al. (designated 'ChsD' here) (6). 'ChsD', together with *A. fumigatus* ChsE, lacks the myosin motor-like domain and has a C-terminus different from that of CsmA (8, 18). The ORF of '*chsD*' (1490 a.a.) might be a partial sequence of CsmA. However, we could not deny the possibility that shorter transcripts could be produced at lower level from one gene.

DISCUSSION

The co-existence of chitin synthase and a myosin motor-like domain in the same molecule of CsmA provides a new insight into the transport and localization of its enzymatic activity. In yeast, many genetical results have shown that chitin distribution is affected in cells of aberrant actin cytoskeleton (3). To add this, our preliminary data suggests that the N-terminal domain of CsmA possesses critical roles in the spatial control of cell wall synthesis (unpublished results). We speculate the N-terminal domain could be active as a myosin motor, and that it would be necessary for proper localization of the C-terminal chitin synthase domain. CsmA as well as other chitin synthases has transmembrane domains, so it might also be possible that CsmA functions as a carrier of the CsmA-containing membrane vesicles.

ACKNOWLEDGMENTS

We thank Dr. Tomas Zimmer for helpful suggestions. We also thank Dr. Kazuo Sutoh for critical reading of the manuscript. This

work is supported by grants from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Bartnicki-Garcia, S. (1968) *Ann. Rev. Microbiol.* **22,** 87–108.
2. Bulawa, C. E. (1993) *Ann. Rev. Microbiol.* **47,** 505–534.
3. Cid, V. J., Duran, A., del Rey, F., Snyder, M. P., Nombela, C., and Sanchez, M. (1995) *Microbiol. Rev.* **59,** 345–386.
4. Bowen, A. R., Chen-Wu, J. L., Momany, M., Young, R., Szaniszlo, P. J., and Robbins, P. W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 519–523.
5. Beth Din, A., Specht, C. A., Robbins, P. W., and Yarden, O. (1996) *Mol. Gen. Genet.* **250,** 214–222.
6. Specht, C. A., Liu, Y. L., Robbins, P. W., Bulawa, C. E., Iartchouk, N., Winter, K. R., Riggle, P. J., Rhodes, J. C., Dodge, C. L., Culp, D. W., and Borgia, P. T. (1996) *Fungal Genet. Biol.* **20,** 153–167.
7. Yanai, K., Kojima, N., Takaya, N., Horiuchi, H., Ohta, A., and Takagi, M. (1994) *Biosci. Biotechnol. Biochem.* **58,** 1828–1835.
8. Motoyama, T., Kojima, N., Horiuchi, H., Ohta, A., and Takagi, M. (1994) *Biosci. Biotechnol. Biochem.* **58,** 2254–2257.
9. Motoyama, T. Fujiwara, M., Kojima, N., Horiuchi, H., Ohta, A., and Takagi, M. (1996) *Mol. Gen. Genet.* **251,** 442–450, *corrected* in (1997) **253,** 520–528.
10. Oakley, C. E., Weil, C. F., Kretz, P. L., and Oakley, B. R. (1987) *Gene* **53,** 293–298.
11. Rowlands, R. T., and Turner, G. (1973) *Mol. Gen. Genet.* **126,** 201–216.
12. Mellado, E., Aufauvre-Brown, A., Specht, C. A., Robbins, P. W., and Holden, D. W. (1995) *Mol. Gen. Genet.* **246,** 353–359.
13. Mooseker, M. S., and Cheney, R. E. (1995) *Ann. Rev. Cell Dev. Biol.* **11,** 633–675.
14. Hasson, T., and Mooseker, M. S. (1994) *Curr. Opin. Cell Biol.* **7,** 587–594.
15. Smith, C. A., and Rayment, I. (1996) *Biophys. J.* **70,** 1590–1602.
16. Montell, C., and Rubin, G. M. (1988) *Cell* **52,** 757–772.
17. Reinhard, J., Scheel, A. A., Diekmann, D., Hall, A., Ruppert, C., and Bahler, M. (1995) *EMBO J.* **14,** 697–704.
18. Aufàuvre-Brown, A., Mellado, E., and Holden, D. W. (1997) *Fungal Genet. Biol.* **21,** 141–152.