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IDENTIFICATION AND CHARACTERIZATION OF POLYUBIQUITIN GENE FROM cDNA LIBRARY OF ASPERGILLUS FUMIGATUS

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ABSTRACT

Aspergillus fumigatus (Afu) causes allergic and invasive forms of diseases in humans. In order to identify genes relevant for pathogenesis, a total of 235 cDNA clones were randomly selected and sequenced from cDNA library of Afu. One of the partially sequenced cDNA clones was homologous to polyubiquitin. Sequencing of the complete cDNA clone showed an open reading frame of 912 bases. Comparison with genomic sequence of Afu using BlastN program, revealed that polyubiquitin gene comprises of 992 bases and contains one intron of 80 bases. The recombinant expression of fusion protein showed an approximately molecular weight of 43-kDa on SDS-PAGE. The translation product of the cDNA sequence showed four tandem repeats of 76 amino acid residues in a single polyubiquitin protein and showed 100% identity with polyubiquitin protein sequences of S. cerevisiae, N. crassa, C. albicans, S. pombe, and M. grisae. Polyubiquitin gene is known to play important role in a variety of cellular processes and recently have been implicated in fungal pathogenesis. Identification of polyubiquitin gene of Afu has opened up scope to study its role in understanding Aspergillus biology and pathogenesis.

KEY WORDS

Aspergillus fumigatus, Polyubiquitin and cDNA.

INTRODUCTION

Aspergillus is an important genus of microorganisms. Among the 182 recognized species (1), Aspergillus fumigatus (Afu) is the most common human pathogen where as Aspergillus flavus is unique in being both plant and human pathogen. Aspergillus parasiticus is a producer of aflatoxin, major mycotoxin in the foodstuffs such as groundnut, maize etc leading to economic losses to the country (2). Afu, a ubiquitous pathogen, causes allergic and invasive forms of diseases depending upon the host immune systems (3). Little is known about the genes/ proteins involved in the pathogenesis of Afu and production of secondary metabolite in the microorganism.

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Till now 828 proteins of Afu have been identified and about 1036 gene sequences have been reported in National Center for Biotechnological Information (NCBI). Recently the genome sequencing for Afu has been completed and is available at The Sanger center and The Institute for Genomic Resources (TIGR). Sequencing of cDNA clones from cDNA libraries and identification of the functions is one of the important methods to characterize genes. This approach had earlier proved useful in the identification of new proteins and enzymes synthesized by the pathogenic and non-pathogenic organisms (4-12). Comparative analysis of genes of Aspergillus species and other fungi can lead to the identification of virulent factors relevant to pathogenesis. So far through random & immunoscreening of cDNA library from our laboratory, a catalogue of 125 Expressed sequence tags (ESTs) of Afu have been prepared for the first time from India. (13-14). Several functionally important ESTs of Afu were identified, such as genes of ubiquitin family, glyoxylate pathway and polyketide pathway (10).

In the present study we completely sequenced the cDNA clone for polyubiquitin gene of Afu for the first time, identified polyubiquitin gene structure and sequence from Afu whole genome shotgun assembly available

at Sanger center and compared putative translated cDNA sequence with polyubiquitin protein sequence of other fungi.

MATERIALS AND METHODS

Isolation and random sequencing of cDNA clones. A cDNA library made from mycelial extract of Afu, grown at 37 oC was obtained from Stratagene (La Jolla, CA, USA). The library was expanded in E. coli XL-1 Blue MRF' cells according to the manufacturer's instructions. The Uni-ZAP XR lambda cDNA clones in the library were converted to pBluscript SK (+) phagemid by in vivo excision as described by the manufacturer. The E. coli SOLR cells were infected with the phagemid and were plated onto LB-ampicillin (50 (g/ ml) agar plates and incubated at 37 oC overnight. Colonies appearing on the plate containing the phagemid having the cDNA insert were used for polymerase chain reaction (PCR) using T3 (5' AATTAACCCTCACTAAAGGG 3') and T7 (5' CGGGATATCACTCAGCATAAT 3') primers. PCR cycling conditions were 94 oC/ 4 min and 28 cycles of 94 oC/ 1 min, 58 oC/ 1.5 min, 72 oC/ 2 min, followed by a terminal extension cycle at 72 oC/ 7 min. PCR amplification product was purified with GFXTM PCR and Gel band purification kit (Amersham Pharmacia Biotech Inc.) and sequenced using T3 primer by ABI-377 DNA Sequencer (Applied Biosystems). The partial cDNA sequence (475 bases) showing sequence similarity with polyubiquitin was further sequenced using reverse primer T7. The sequence obtained from T3/T7 was overlapped and complete cDNA sequence for polyubiquitin was identified. To identify complete gene sequence, it was subjected to BlastN against Afu whole genome shotgun assembly at Sanger institute (www.sanger.ac.uk/ projects/A_fumigatus) and putative function and comparative analysis of polyubiquitin was carried out as described in Shankar et al 2004 (10).

Restriction digestion and recombinant expression of polyubiquitin gene. The SOLR E. coli cells harboring the pBluescript SK (+) plasmid were used for plasmid isolation and sub- cloned cDNA insert was double digested by the protocol as described in Sambrook et al 1989 (15). The recombinant expression of protein was carried out as described in Nigam et al 2001 (14). In short, the SOLR E. coli cells harboring the phagemid were inoculated into LB broth with 50 g/ml of ampicillin and incubated at 37 °C until the absorbance (A600) reached 0.2. The fusion protein was induced with 1.0 and 5.0 mmol/l IPTG and the cultures were further grown for 5 h. The cell pellet obtained after centrifugation at 1600×g for 15 min at 4°C was suspended in 50 mM Tris-Cl (pH 7.5) and sonicated. After centrifugation at 16,000×g for 45 min at 4 °C, the supernatant was separated. The supernatant containing the recombinant expressed protein was analyzed on 12% polyacrylamide gel under reducing and denaturing conditions.

RESULTS AND DISCUSSION

Ubiquitin (UBI), a 76 amino acid polypeptide, is highly conserved in evolution and present in all eukaryotic organisms. It exists in cells as free and also in conjugated forms. The covalent ligation of UBI to various receptor proteins in eukaryotic cells results in or regulates a number of cellular process, such as selective protein degradation, DNA repair, progression through the cell cycle, signal transduction, transcriptional, the nuclear transport process, receptor control by endocytosis, the processing of antigens in the immune system, pathological alterations and programmed cell death (15-17).

The major function of UBI is to label proteins destined for selective elimination. UBI-encoding sequences can be divided into two groups. The polyubiquitin gene encodes a protein containing tandem head-to-tail repeats of the monomeric UBI sequence. Polyubiquitin then processed by de-ubiquitinating enzymes to produce its monomers. The other class of UBI genes encodes a terminal UBI sequences joined to one of two ribosomal proteins also referred to carboxyl extension proteins (CEP) (18). The CEP is then released from the fusion proteins in vivo by the action of de-ubiquitinating enzymes. A cDNA library made from mycelial extract of Afu grown at 37 °C was obtained from Stratagene. From the 154-cDNA sequences, a

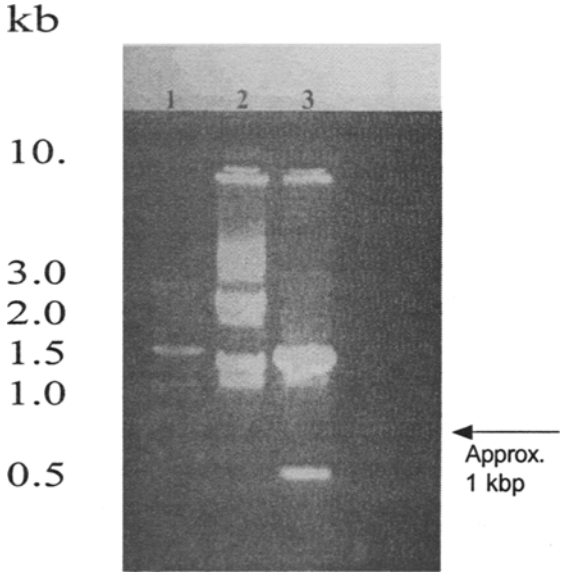

Fig- 1. 1% Agarose gel in TAE buffer showing size of cDNA clone of length 1kb after double digestion with EcoRI and XhoI.

Lane-1 1kb DNA ladder;
Lane-2 pBluescript phagemid;
Lane-3 pBluescript digested with EcoRI and XhoI.

atg cag att ttc gtc aag act ctc acg ggc aag acc atc acc ttg gag gtc gag tcc agc gac act
Met Q I F V K T L T G K T I T L E V E S S D T
att gac aat gtc aag tcg aag att cag gat aag gag ggt atc cct cct gac cag cag cgc ttg att ttc
I D N V K S K I Q D K E G I P P D Q Q R L I F
gct ggc aag cag ctc gag gat ggc cgc act ctt tcc gat tat aac atc cag aag gaa tct act ctg
A G K Q L E D G R T L S D Y N I Q K E S T L
cac ctg gtt ctc cgt ctt cgt ggt ggc **atg** caa atc t GTAAGTTACTTT GCCTTCTGCCTA
H L V L R L R G G **Met** Q I
AT TTTGCCACCCTGGTGGCCACATTTTAG GGT CGCCCGCTGACTCAT TG
ATAACAG tc gtc aag acc ctc act ggc aag acc att act ctc gag gtg gaa tcc agc gat acc
F V K T L T G K T I T L E V E S S D T
att gat aac gtt aag tcg aag atc cag gac aag gag ggt atc cct cct gac cag cag cgt ctg att ttc
I D N V K S K I Q D K E G I P P D Q Q R L I F
gct ggc aag cag ttg gag gat ggc cgc acc ttg tcc gac tat aac att cag aag gaa tcg act ctc
A G K Q L E D G R T L S D Y N I Q K E S T L
cac ctc gtc ctt cgt ctt cgt ggt ggt **atg** caa atc ttc gtt aag act ctc acg gga aag act atc aca
H L V L R L R G G **Met** Q I F V K T L T G K T I T
tta gaa gta gag tct tca gac acc atc gac aat gtc aag agc aag atc cag gat aag gag ggt atc
L E V E S S D T I D N V K S K I Q D K E G I
cct cct gat caa cag cgt ctc atc ttt gct gga aag caa ctt gaa gat ggc cgt acc ttg tct gat tac
P P D Q Q R L I F A G K Q L E D G R T L S D Y
aat atc caa aag gaa tcc aca ctg cat ctg gtc ctc cgt ctg cgt ggt ggc **atg** cag atc ttc gtc
N I Q K E S T L H L V L R L R G G **Met** Q I F V
aag act ctc acc gga aag acc att acc ctg gag gtg gag tcc tcc gac acg att gac aac gtg aag
K T L T G K T I T L E V E S S D T I D N V K
agc aag atc caa gac aag gag ggc atc cct cct gac cag cag cgt ctc atc ttc gct ggt aag cag ttg
S K I Q D K E G I P P D Q Q R L I F A G K Q L
gaa gat ggg agg acc ctc tcc gat tac aat att caa aag gag agc acg ctc cac ctg gtg ctg cgt ctg
E D G R T L S D Y N I Q K E S T L H L V L R L
cgt ggt gga
R G G

Fig-2 Sequence of the polyubiquitin gene and predicted translation product. Intronic nucleotides and Amino acids are shown in uppercase letters.

partially sequenced cDNA clone showed sequence similarity with polyubiquitin gene. Sequencing of the complete cDNA clone revealed an open reading frame of 912 bases. This cDNA clone was showing a size of length of less than 1kilo bases after double digestion with EcoRI and XhoI (Fig-1). On comparing with genomic sequence of Afu using BlastN program displayed that 992 bases of polyubiquitin gene contains one intron of 80 bases (Fig-2). The identified gene sequence of polyubiquitin was submitted to GenBank (AY817687). When the cDNA clone showing sequence similarity with polyubiquitin gene is subjected to BlastN, showed significant sequence similarity at different contigs of Afu genomic sequences with variable expect values, suggesting that it may be copies of the same genes with similar function. The translation product of the cDNA sequence showed four tandem repeats of 76 amino acid residues in a single polyubiquitin protein (Fig-2). The cDNA sequence showed homology when subjected to BlastN with fungi at NCBI with polyubiquitin genes of Aspergillus nidulans (Identity=83%), Magnaporthe grisea (82%), with Neurospora crassa (83%) and with Schizosaccharomyces pombe (78%). The

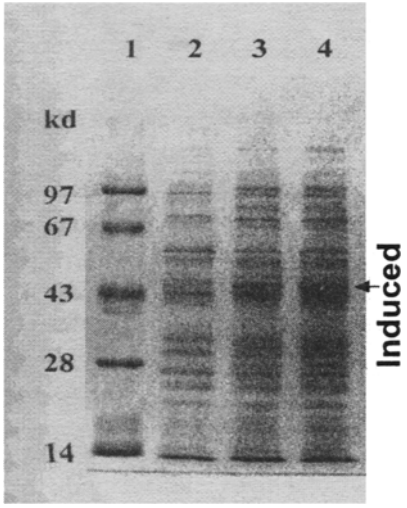

Fig- 3 SDS- PAGE (12%) stained with co-omassie brilliant blue R-250 showing expression of recombinant from a cDNA clone at different concentration of IPTG.

Lane -1 Molecular weight marker in kDa;
Lane-2 Uninduced E. coli cell lysate;
Lane-3 Induced E. coli cell lysate (1mM IPTG);
Lane-4 Induced E. coli cell lysate (5mM IPTG).

recombinant expression of fusion protein showed an approximately molecular weight of 43-kDa on SDS-PAGE (Fig-3). When deduced amino acid sequence of identified cDNA sequence is subjected to BlastP at non-redundant protein database of NCBI; it showed 100% identity with polyubiquitin protein sequences of S. cerevisiae, N. crassa, C. albicans, S. pombe, and M. grisae. Thus polyubiquitin protein is highly conserved and UBI fusion proteins are apparently interchangeable in function due to the conjugation of ligated protein (11). The three dimensional structure of Ub reveals that the residue Glu64 is located close to the N terminal sequence surrounding the surface phe4. Further, N terminal fragment of ubiquitin (residues 1-34) adopts a Beta hairpin (residues 1-17), followed by an alpha helix (residues 23-34), which may be important for the interaction with membranes. It has been reported that various cell wall proteins (receptors) of C. albicans interacting with matrix proteins, such as 37-kDA laminin receptors, the 58 kDa fibrinogen-binding mannoprotein, and the candidal C3d receptor, are polyubiquitinated (19). Similar receptor proteins have also been reported from Afu (20,21).

Hence, ubiquitin may play a role in modulating the activity of these receptors and the interaction of spores

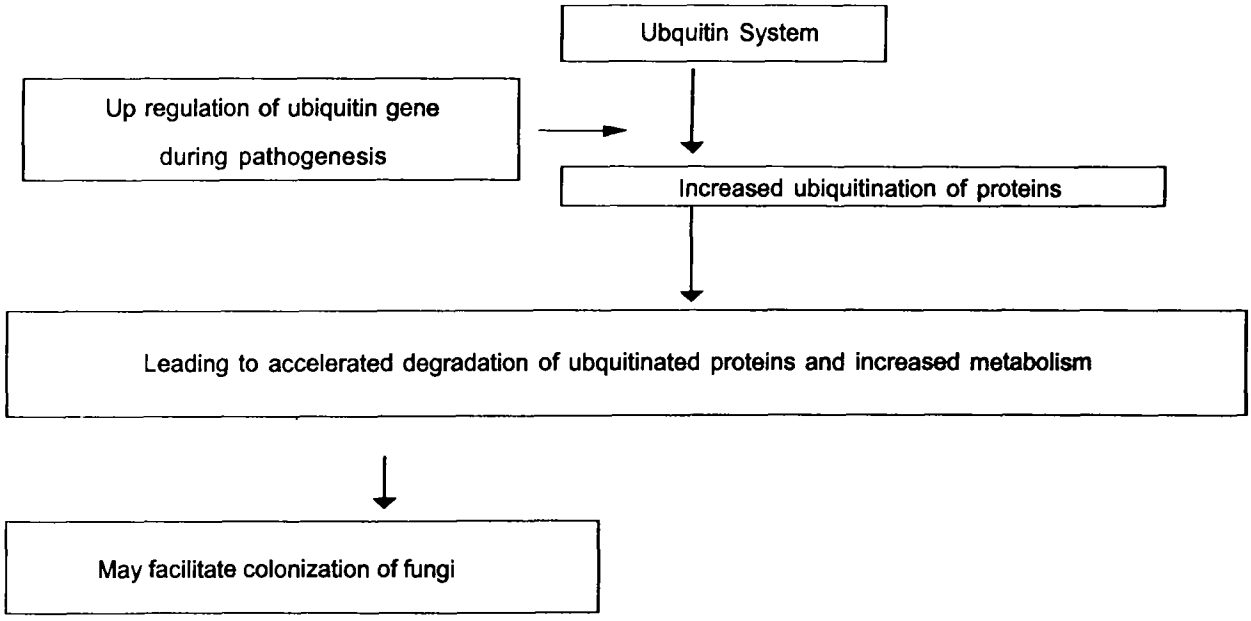

Fig-4 Ubiquitin system -Disease

Ubquitin System
Up regulation of ubiquitin gene during pathogenesis Increased ubiquitination of proteins Leading to accelerated degradation of ubquitinated proteins and increased metabolism
May facilitate colonization of fungi

of Afu with the host matrix proteins structure as in the case of C. albicans. Further, genes of polyubiquitin family are expressed at higher level during colonization of rice leaves by the pathogenic fungus Magnaporthe grisea and in the human pathogen Paracoccidioides brasiliensis. A schematic diagram of role of ubiquitin system in disease condition is represented in the figure-4. Current study opens the scope for understanding the role of ubquitination of proteins that may be leading to the pathogenesis of Afu.

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