

9. Studies on diarrhoeagenic parasites

9.1 Cloning and Characterization of Collagenase genes of *Entamoeba histolytica*

Investigator: S. Ganguly

Isolated electron dense granules (EDG) were characterized by biochemical and immunological parameters. The purified EDG showed 8 times more collagenolytic activity than the whole *E.histolytica* trophozoites. Purified EDG showed six polypeptide bands with apparent molecular weights of 108, 106, 104, 97, 68 and 59 kDa that were not detected in whole *E.histolytica* extracts. Similarly, two protease activities with apparent molecular weights of 40 and 85 kDa were detected only in EDGs. Scanning transmission electron microscopy clearly demonstrated that EDGs were highly complex molecule, mainly made of cations. Collagenolytic activity from EDG has been purified by FPLC MONO-Q HR 5/5-matrix and maximum activity was observed in fraction 16. However, no further functional studies could be conducted because, the purified enzyme was highly unstable. To overcome the problem several approaches were made. One that is working better is solubilization of EDG, using 1% Triton X-100 and demonstration of collagenolytic activity in the soup. The confirmation of collagenolytic activity of the soluble fraction was done by zymography analysis. A broad single clear zone compared to parent EDG in soluble fraction was seen at 85 kDa by zymography technique.

To purify the collagenolytic activity, the solubilized fraction from EDG has been applied on to the size exclusion matrix (sephacryl 300). Fractions were collected and activity was tested by zymography in polyacrylamide co-polymerized collagen gel. Five fractions showed collagenolytic activity. These fractions were further purified by ion exchange chromatography. Purified fractions were collected and activity was once again tested by zymography. This purified fraction was transferred to PVDF membrane and send for sequencing.

Antibody raised against EDG was also used in screening a pathogenic *E. histolytica* cDNA library constructed in λ ZAP plus II vector. Ten plaques showing intense colour were digested with EcoRI and XhoI to release insert. Sequencing and BLAST search analysis of plasmid DNA of ten clones revealed homology with four genes in GenBank viz. Actinin like protein (ALP) (Acc.No. AF208390), Grainin 1 (Acc.No. AF085196), Serine rich protein (Acc.No. M34438), gEh29 gene for alkyl-hydroxperoxidase reductase (Acc.No. X70996). Primers were designed to get the full-length gene coding for grainin I to study its function and localization in EDG. Clone PMLrfr5 representing the full-length protein (Grainin I) was digested with BamHI and HindIII and the released fragment was ligated to pMALC2X expression vector and transformed into competent *E. coli* XLI Blue cells. Plasmid from the positive clone having in frame orientation with the vector sequence was confirmed by sequencing. The right orientated plasmid was then transformed into *E. coli* BL21 (DE3) competent cells. Transformed cells were screened by blue white screening on LA plate containing ampicillin and one of them harboring pMALC2X-grainin DNA was selected for recombinant protein expression.

In optimization experiment, LB 0.6 OD, 0.6 mM IPTG concentration, 6 hours, 26°C was optimal in expression of recombinant protein.

The recombinant protein was purified from soluble fraction using maltose affinity chromatography column as a maltose-tag fusion protein. The recombinant protein was eluted with 5 volumes of elution buffer containing 20mM maltose. Over expressed band was appeared in 64 kDa in 10% SDS-PAGE analysis. After expression, affinity purification and proteolytic removal by factor-Xa protease of maltose binding protein, bands were appeared at 42kDa and 22kDa in commassie stained SDS-PAGE. This 42kDa and 22kDa protein represents the maltose binding and Grainin 1 protein respectively. Antibody was raised against Grainin 1 protein and used for localization and immunoblot assay. The localization and co-localization experiments clearly demonstrate that Grainin 1 protein is present within the electron dense granules. The antibody against the recombinant purified protein showed a single immunoreactive band at 22kDa position against both in EDG and recombinant protein, thereby confirming that recombinant protein has similar epitopes with that present in the EDG.

Co-immunoprecipitation assay:

Proteins from solubilized EDG were immunoprecipitated with purified anti-grainin1 antibody and preimmune sera as a negative control. Protein A-sepharose with covalently bound IgG and immunoprecipitated protein was pelleted and was with wash buffer for 3 times. The pellet was resuspended in sample buffer and analyzed by 10% SDS-PAGE along with the whole cell extract which served as a positive control. Proteins were transferred in nitrocellulose membrane and developed with anti P²¹ activated kinase antibody at 1:1000 dilution.

A protein of 53kDa (fig1) was specifically recognized by anti P²¹ activated kinase antibody.

Co-localization study of PAK 1 & Grainin1:

To examine whether Grainin 1 protein co-localizes with P²¹ activated kinase that are known to be associated with the *Entamoeba histolytica* cytoskeleton during phagocytosis, we performed double staining of trophozoites with antibody against Grainin1 and P²¹ activated kinase antibody. FITC labeled P²¹ activated kinase was visualized green and rhodamine labelled grainin 1 was visualized red. Grainin 1 was found to co-localize in cytoplasm and phagocytic cup with P²¹ activated kinase (fig 2). The yellow spot indicated that P²¹ activated kinase and Grainin 1 protein were colocalized. This result suggests that P²¹ activated kinase and Grainin1 protein interacting each other.

Immunofluorescence labeling with actin:

To examine whether Grainin 1 protein co-localizes with other cytoskeleton protein other than P²¹ activated kinase that are known to be associated with the *Entamoeba histolytica* cytoskeleton during phagocytosis. To know that we performed double staining of trophozoites with antibody against Grainin1 and actin antibody.

This grainin 1 protein was colocalized with actin protein within membrane extension and in cytoplasm (fig 3).

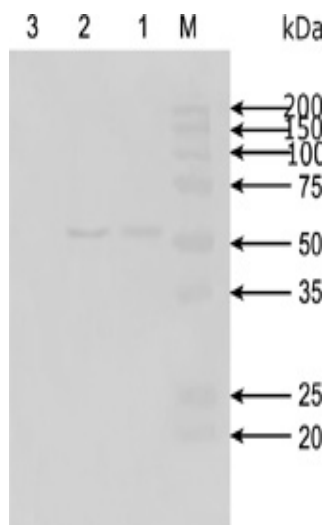


Fig 9.1.1: Co-immunoprecipitation of Grainin 1 protein with Electron Dense Granules. Lane 1) Co-immunoprecipitated protein, Lane 2) Electron Dense Granules (3) Co-immunoprecipitated protein with Pre-immunoserum, Lane M) Molecular weight marker.

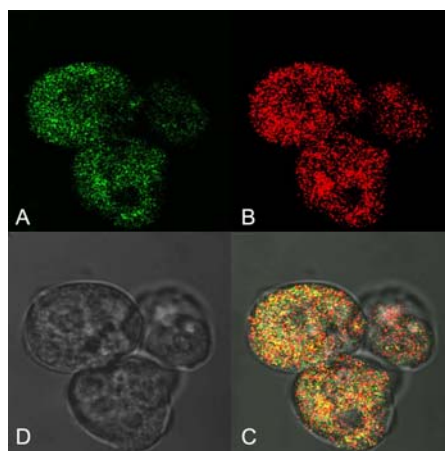


Fig 9.1.2: Co-localization of Grainin 1 protein with P^{21} activated kinase inside *E. histolytica* cell. Grainin 1 protein is stained with TRITC and P^{21} activated kinase stained with FITC A) FITC stained P^{21} activated kinase protein; B) TRITC stained Grainin 1; C) Co-localization of Grainin 1 protein with P^{21} activated kinase; D) Phase contrast picture of *E. histolytica* whole cell.

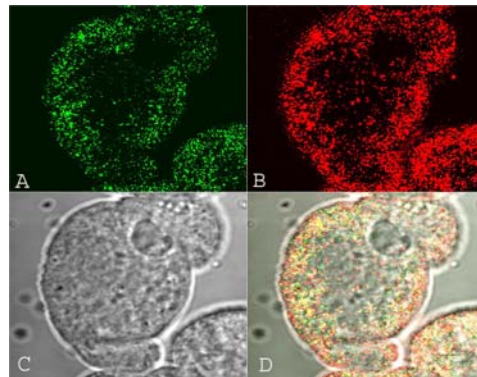


Fig 9.1.3: Co-localization of Grainin 1 protein with actin inside *E. histolytica* cell. Grainin 1 protein is stained with TRITC and actin stained with FITC. A) FITC stained actin; B) TRITC stained Grainin1; C) Phase contrast picture of *E. histolytica* whole cell, D) Co-localization of Grainin 1 protein with actin.

9.2 Studies on RNA maturation and processing in *Giardia lamblia*

Investigator: S. Ganguly

Giardia lamblia, a binucleate flagellated intestinal protozoan parasite, is a frequent cause of both epidemic and endemic diarrhoeal illness in developed and developing countries. Infection of *Giardia* in human shows various clinical signs. There may be total latency, acute short lasting diarrhoea, or chronic syndromes associated with nutritional disorders, weight loss and failure to thrive. Till date drugs viz. Metronidazole, tinidazole, nitromidazole, quinacrine and furazolidine are used for treatment of giardiasis.

Giardia lamblia is regarded as one of the most conserved eukaryotes evolved from the prokaryotes. In eukaryotes rRNA processing was found to involve small ribonucleoprotein particles containing different nuclear proteins and small nucleolar RNAs (snoRNAs) in the nucleolus. Mature small RNA species are almost entirely (>95%) nuclear. These small nucleolar RNAs (snoRNAs) have been described to have unique sequences (Box C/D or H/AC conserved) and interact directly with unique segments of pre-rRNA *in vivo*. Transfection of antisense oligodeoxynucleotides have shown specific degradation of these snoRNAs. Specific disruptions of prerRNA processing were then observed, which were reversed by transfection of the corresponding *in vitro*-synthesized snoRNA suggesting their crucial role in pre-rRNA processing. In *Giardia lamblia* no nucleolus has been detected until today. Although presence of some nuclear proteins like fibrillarin and small nuclear RNAs (snRNAs) suggests that there might be a similar type of ribosome biogenesis and modification in this parasite. The ribosomal RNA gene of this parasitic protozoan has been chosen to understand the molecular mechanism of its processing for future development of therapeutic intervention. The current project was undertaken with an objective to study the maturation process of rRNA and its conserveness with human system which will help to develop a clear idea in post transcriptional modification mechanism of rRNA in this protozoa and hence to establish newer, specific and selective potential targets for drugs.

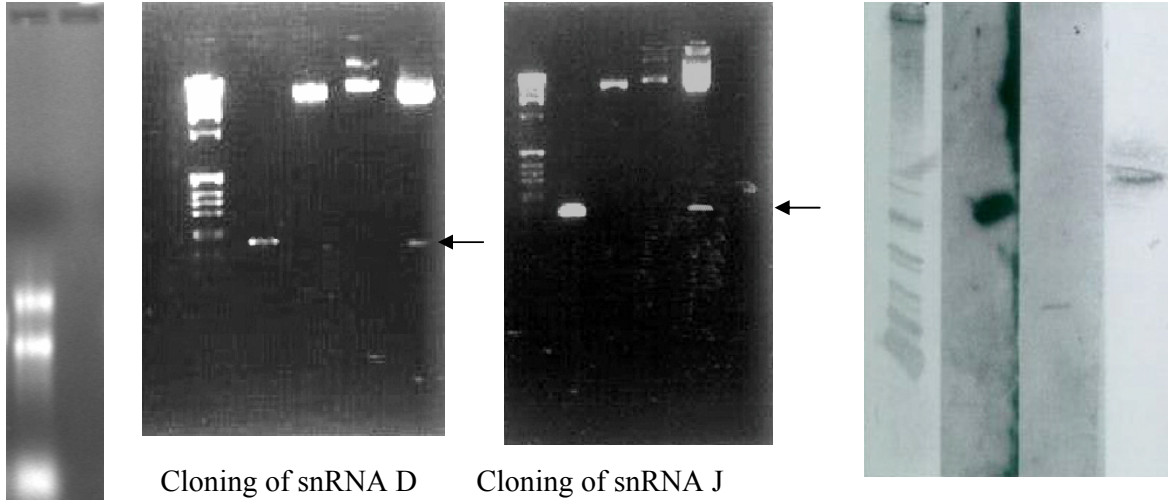
- Axenic maintenance of different *Giardia lamblia* strains have already been carried out.
- Two Giardial snRNA has been cloned by RTPCR followed by *in vitro* transcription of hot radiolabelled snRNA.
- Nuclear extract of *G. lamblia* has been made and incubated and UV cross linked with hot snRNA followed by Rnase protection assay.
- The UV cross linking study is again performed with competition of cold snRNA to identify specific new proteins involved in cross linking with snRNA and complex formation.
- Several new specific protein bands have been identified to participate in binding. Out of them one band was found to be fibrillarin as suggested by western blotting analysis.
- Recombinant clone of *Giardia* fibrillarin has been raised and further subcloned in suitable expression vector for easy purification.
- Fluorophore tagged snRNA preparation is under progress to identify the localization of snRNAs *in vivo*.
- Transfection and electroporation of labeled snRNA into *Giardia* cells are also under progress for *in vivo* identification of the proteins involved in binding.

- Construction of *Giardia lamblia* genomic DNA microarray is under progress. To achieve this goal, first a genomic DNA library is needed. We are in this stage where genomic DNA has been isolated from the parasite. Then the total genomic DNA has been sheared in desired fragments (1-1.5kb). These sheared DNAs are then cloned to make a genomic DNA library. This library will be further used in array spotting to make a DNA chip for *Giardia*.

Figures:

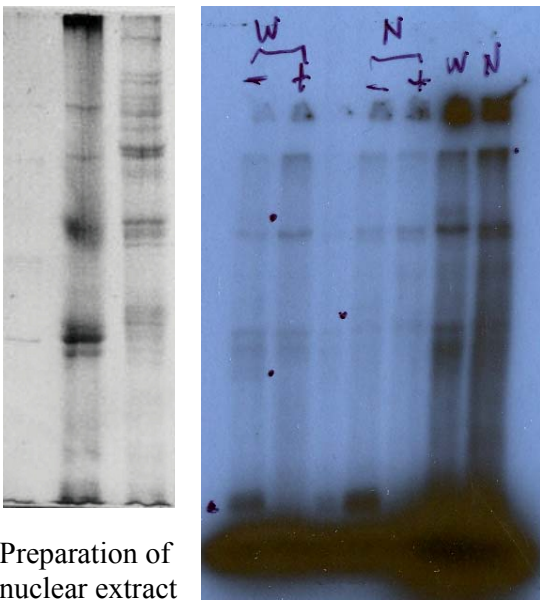
Primers for cloning snRNA D and J

SG5 - 5' - TTTTAAGCTTGTCTAGACGCGTCCTGG-3'	5' terminal primer_____	81°C
SG6 - 5' - TTTTGAATTCAAGGACTATAGGGGCGGT-3'	3' terminal primer_____	85°C
SG7 - 5' - TTTTAAGCTTCTGCCTCTCCTGAAGCAG-3'	5' terminal primer_____	87°C
SG8: - 5' - TTTTGAATTCAGAATACGACAA-3'	3' terminal primer_____	61°C



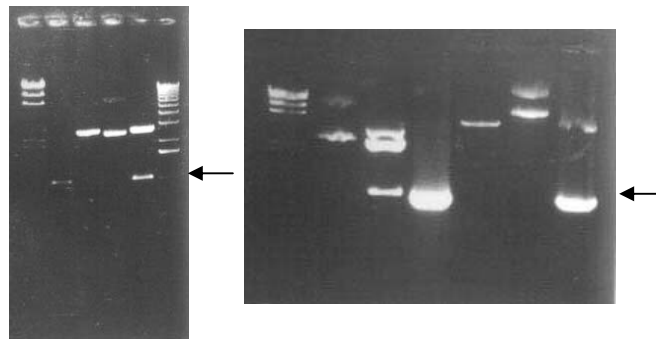
Isolation of total RNA of *G. lamblia*

Northern Hybridization



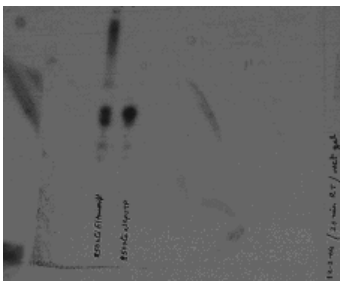
Preparation of nuclear extract

UV cross linking followed by RNase protection assay



Cloning of fibrillarin in pGEM4Z and pET33b(+)

Shearing of genomic DNA using nebulizer : lane 1 – marker, Lane 2 – Sheared genomic DNA (1-1.5 Kb) of *Giardia lamblia*.



In vitro transcription of snRNAs