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by Lalu Rudyat Telly Savalas C17

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Construction and Expression of Human Disrupted in Renal Carcinoma 2 (DIRC2) in *Escherichia coli*

Lalu Rudyat Telly Savalas*[1,2], An Nuurun Nisa[2], Handa Muliasari[2] and Yunita Arian Sani Anwar[1,2]

[1] Department of Mathematics and Sciences Education, University of Mataram
[2] Biosciences and Biotechnology Research Group, University of Mataram
Jl. Majapahit No. 62 Mataram 83125
*email: telly@daaad-alumni.de

Abstract

Re-exporting process of recycled materials from the lumen of lysosomes into cytosol involves lysosomal transmembrane proteins whose defect may lead to mild to severe diseases. Due to the difficulty in the study of transmembrane proteins, our current knowledge of transmembrane proteins, especially in organelle transmembrane proteins are still limited. Disrupted in renal carcinoma 2 (DIRC2), has been recently identified as a constituent of lysosomal transmembrane protein. Structurally, the protein belongs to a member of major facilitator superfamily (MFS), a large group of secondary tranporter proteins with diverse substrates and characterized by the presence of, but not limited to, twelve transmembrane spanning domains. DIRC2 shows an unique characteristic that it is fragmented into two nearly equal fragments in the lysosomal fraction of mammalian cells. *In vivo* study has revealed that cathepsin L is likely to be involved in the proteolysis of DIRC2. In order to analyze the possibility DIRC2 is directly processed by cathepsin L, DIRC2 gene from human needs to be constructed and be expressed in full length within *Escherichia coli*, which is a comfortable host for heterologous protein expression and has been proven to work well in expressing transporter protein of major facilitator superfamily. Human DIRC2 gene was PCR amplified and ligated with pGEX-2T plasmid. The resulted construct was used to transform *E. coli* BL21 (DE3) which has an overexpression system for gene placed downstream to its T7 promotor, under induction of isopropyl β-D-1thiogalactopyranoside (IPTG). Expression of DIRC2 in *E. coli* is discussed and the over-expressed full length DIRC2 provides a prerequisite material to investigate its proteolysis by cathepsin L.

Keywords: DIRC2, transporter protein, *E. coli* transformation, heterologous expression

Introduction

Disrupted in renal carcinoma 2 (DIRC2) has been recently described in the context of genetic study of patient with inherited renal carcinoma (Bodmer et al 2002). This protein has later been described as a putative constituent of human lysosomal membrane (Schroeder et al 2007). Bioinformatics analysis of this protein revealed that this protein belongs to member of major facilitator superfamily, a family of transporter protein which plays a role in diverse transport activities across biological membrane.

Most recent biochemical study of DIRC2 shows an intriguing property of this transporter protein that it is expressed as a fragmented protein. Although membrane protein

fragmentation is a known phenomenon, fragmentation of a major facilitator superfamily transporter protein has not been previously reported. Study by Savalas and co-workers (Savalas et al 2011) has underlined a putative role of cathepsin L in the processing of DIRC2. Lysosomes are known to be the host of tenth of hydrolases/proteases. However, it is not clear whether cathepsin L directly cleaves DIRC2. Moreover, the putative processing site of DIRC2 is not a usual cleavage site of cathepsin L. An alternative to the direct cleavage of DIRC2 by cathepsin L is the indirect process, where an unknown protease is initially activated by cathepsin L which subsequently cleaves DIRC2.

Due to the enormous stability of DIRC2 fragments in lysosomes membrane, it is unlikely that the processing of DIRC2 lead to the degradation pathway. Instead, the processed form of DIRC2 may still exert (or even be required for) its activity. If the later scenario is true, both fragments of DIRC2 have to be tightly bound to each other. In the present work, attempts will be made to show express human DIRC2 in a bacterial system that lacks of cathepsin L activity with the hope to be able to produce full length DIRC2, and subsequently analysis its processing by cathepsin L *in vitro*.

Material and Method

DIRC2 gene was amplified by using SensoQuest thermal cycler. The primer pair used was 5'-TATAGGATCCATGGGCTCTCGCT-3'(forward) and GCTGAATTCTTAAAC GGAGACAACCAC-3'(reversed) that has *Bam*HI and *Eco*RI cleavage sites, respectively. The 1437 bps fragment was analyzed and eluted from 1% agarose gel (Thermo Scientific). Amplicon and expression vector pGEX-2T were double digested with *Bam*HI and *Eco*HI (TaKaRa) and both, following restriction reaction, were gel-purified from 1% agarose gel. Ligation was undertaken by incubating linearized plasmid pGEX-2T and DIRC2 fragment together with T4 DNA ligase overnight at 17^{o}C. Ligation was stopped by incubation at 65^{o}C for 10 minutes. Ligation mix was used to transform *E. coli* XL1-blue by using an electroporator (Bio-Rad) and the cells were allowed to express their ampicillin resistance gene for one hour prior to spreading onto LB ampicillin plates. Colony-PCR was undertaken for bacteria clones able to grow on LB ampicillin plate. Further confirmation was undertaken by restriction analysis and sequencing of recombinant plasmids. Recombinant plasmid that bear DIRC2 gene was used to transform *E. coli*BL21(DE3) and overexpression in BL21(DE3) under IPTG induction will be carried out.

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Results and Discussion

Results

Sub-cloning of human DIRC2 amplification

Human *DIRC2* gene, 1453 bp, has been amplified by the primer pair described in Materials and Method section. Prior to ligation, the resulted PCR fragment as well as cloning vector pGEX-2T was simultaneously digested with *Bam*HIdan*Eco*RI to produce compatible ends of PCR fragment and pGEX-2T vector. Ligation mix was first used to transform XL1-blue strain of *E. coli*, instead of directly used to transform the BL21(DE3) expressing host. This allowed recovery, and further amplification, of limited recombinant plasmid resulted from low efficiency ligation process.

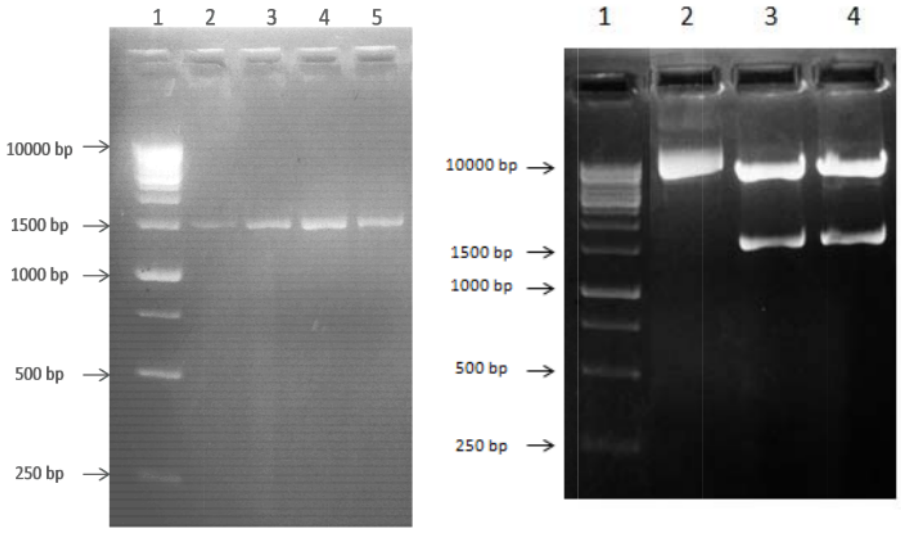

Figure 1. Left: colony-PCR of XL1-blue transformant cells. Line 1: 1 kb marker from Thermo Scientific. Molecular weights are indicated. Lines 2-5 are positive clones that show DIRC2 fragmentsof 1453 bps. Right: Excision of DIRC2 insert from two colonies of *E. coli*BL21(DE3) transformant cells (line 3 and 4). Line 2 shows undigested recombinant plasmid.

Restriction analysis

Recombinant plasmids were isolated from transformed XL1-blue colonies and subjected to restriction analysisi with *Bam*HI and *Eco*RI. Following the double digestion, the digestion mixtures were separated on agarose gel. It is shown that the 1453 base pair size of DIRC2 ORF were released (Figure 1, right).

Sequencing

For further confirmation, the recombinant plasmids weresequenced. Alignment of sequencing results with published sequence of DIRC2 showsno mutation in the loop between transmembrane 5 and 6 (Figure 2), although a few mutation do exist elsewhere but considered insignificant as they are located in the middle of transmembrane domains. Our previous study proved that those regions are not affected by extensive mutation (not shown).

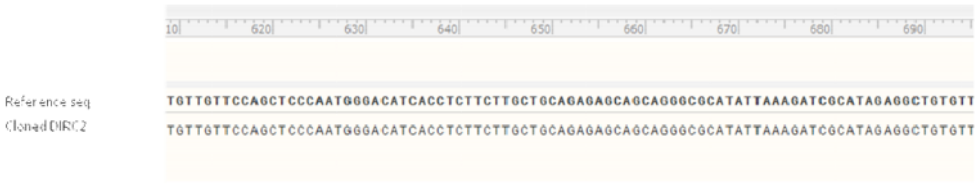

Figure 2.Sequencing result of recombinant plasmid pGEX-2T-DIRC2. The loop region between transmembrane 5 and 6 shows no mutation. (Alignment generated by SnapGene).

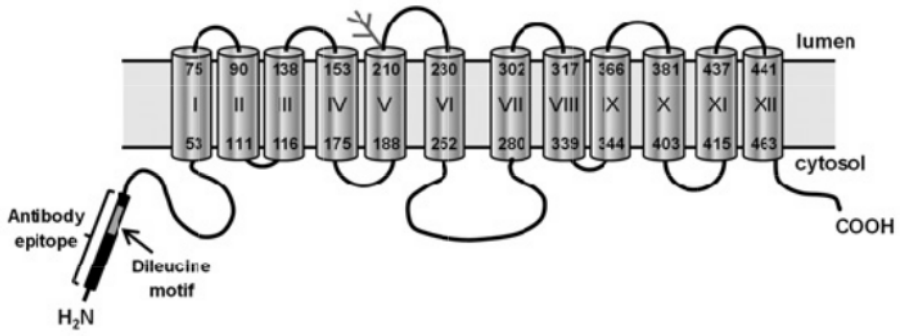

Figure 3.Model structure of DIRC2 protein. Model of DIRC2 was generated by TMHMM server *(*www.cbs.dtu.dk/services/**TMHMM**/). Putative cleavage site of DIRC2 by cathepsin L lies between amino acid 209 to 230 that correspond to base 619 to 690.

Discussion

The intriguing fact shown by DIRC2 when expressed in mammalian cells is that this protein is fragmented into two nearly equal-size halves (Savalas et al, 2011). The nature of this fragmentation is not clear. It may be the entry point of DIRC2 to its degradation, since lysosomes are well-known degradative compartment along with proteasomes system. On the other hand, the stability of both fragments of DIRC2 may be a hint that the cleavage is part of DIRC2 maturation even.

Previous study showed that cathepsin L may directly involve in the proteolysis of DIRC2. The reverse option, i.e.cathepsin L involves indirectly to the processing of DIRC2 is, however, not excluded, since lysosomes are known to be the host of an array of proteolytic enzymes (Saftig and Klumperman, 2009). Additionally, proteolysis networks among lysosomal proteases do exist (reviewed for example by Guha and Padh, 2008). By providing full length form of DIRC2, it is amenable to address the above question, and the cleavage of DIRC2 by cathepsin L is yet to be investigated in our lab.

Conclusion

Human *DIRC2* gene has been successfully amplified using a specific primer pair and subsequently cloned in a bacterial over-expression vector.Alignment shows that there is no mutation in the putative cleavage site region of the DIRC2 protein. The extent to which the gene is able to be over-expressed is currently investigated and the bacteria-based overexpression of human DIRC2 may provide a mean to analyze its proteolysis bycathepsin L.

References

Bodmer, D., Eleveld, M., Kater-Baats, E., Janssen, I., Janssen, B., Weterman, M., Schoenmakers, E., Nickerson, M., Linehan, M., Zbar, B., and van Kessel, A.G. 2002. Disruption of a novel MFS transporter gene, DIRC2, by a familial renal cell carcinoma-associated t(2;3)(q35;q21) *Hum. Mol. Gen.* **11**,641-649.

Guha, S. and Padh, H. 2008.Cathepsins: Fundamental effectors of endolysosmal proteolysis, Indian Journal of Biochemistry and Biophysics, **45**, 75-90.

Law, C.J., Maloney, P.C., and Wang, D.N. 2008. Ins and out of major facilitator superfamily antiporters. *Annu. Rev. Microbiol*., **62**, 289-305.

Saftig, P and Klumperman, J. 2009. Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nat. Rev. Mol. Cell Biol.* **10**, 623- 635.

Savalas, L.R.T., Gasnier, B., Damme, M., Lübke, T., Wrocklage, C., Debacker, C., Jezegou, A., Reinheckel, T., Hasilik, A., Saftig, P. and Schröder, B.A.2011. "Disrupted in renal carcinoma 2" (DIRC2) - a novel transporter of the lysosomal membrane - is proteolytically processed by cathepsin L, Biochem. J., Immediate Publication, doi:10.1042/BJ20110166.

Schroeder, B., Wrocklage, C., Pan, C., Jäger, R., Kösters, B., Schafer, H., Elsässer,H.P., Mann, M., and Hasilik, A.2007. Integral andassociated lysosomal membraneproteins. *Traffic***8**, 1676-1686.

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