Molecular characterization of human ABHD2 as TAG lipase and ester hydrolase


*Department of Biotechnology, K L University, Green fields, Vaddeswaram, Guntur District, Andhra Pradesh 522 502, India

Synopsis
Alterations in lipid metabolism have been progressively documented as a characteristic property of cancer cells. Though, human ABHD2 gene was found to be highly expressed in breast and lung cancers, its biochemical functionality is yet uncharacterized. In the present study we report, human ABHD2 as triacylglycerol (TAG) lipase along with ester hydrolising capacity. Sequence analysis of ABHD2 revealed the presence of conserved motifs G205XS207XG209 and H120XXXXD125. Phylogenetic analysis showed homology to known lipases, Drosophila melanogaster CG3488. To evaluate the biochemical role, recombinant ABHD2 was expressed in Saccharomyces cerevisiae using pYES2/CT vector and His-tag purified protein showed TAG lipase activity. Ester hydrolase activity was confirmed with pNP acetate, butyrate and palmitate substrates respectively. Further, the ABHD2 homology model was built and the modelled protein was analysed based on the RMSD and root mean square fluctuation (RMSF) of the 100 ns simulation trajectory. Docking the acetate, butyrate and palmitate ligands with the model confirmed covalent binding of ligands with the Ser207 of the GXSXG motif. The model was validated with a mutant ABHD2 developed with alanine in place of Ser207 and the docking studies revealed loss of interaction between selected ligands and the mutant protein active site. Based on the above results, human ABHD2 was identified as a novel TAG lipase and ester hydrolase.

Key words: αβ hydrolase domain, ester hydrolase, lipid metabolism, triacylglycerol (TAG) lipase.

Cite this article as: Bioscience Reports (2016) 36, e00358, doi:10.1042/BSR20160033

INTRODUCTION

Lipases in addition to being essential enzymes necessary for the supply of energy also play a major role in lipid signalling and metabolism. Aberrations in lipases were found in many human diseases, including cancer, making it imperative to understand these enzymes. Recently, the mammalian αβ hydrolase domain (ABHD) containing proteins have emerged as novel potential regulators of lipid metabolism and in signal transduction [1]. The human ABHD family contains 21 proteins and is part of a superfamily possessing an αβ hydrolase fold [1,2]. Hydrolase activity of ABHD is attributed to the catalytic triad composed of serine-acid-histidine residues located in loop regions. The corresponding motif found in most of the human ABHD family members was GXSSXG. The acid residue of the catalytic triad can either be a glutamate or aspartate, usually located after strand β7 [3]. Interestingly, the majority of the human ABHD proteins also possess another conserved motif HXXXXD where X is any amino acid residue and the motif was attributed to acyltransferase activity [4].

Some of the human ABHD proteins were well characterized as lipases, including ABHD3 which selectively cleaved medium-chain and oxidatively-truncated phospholipids. ABHD4 was described as lyso-N-acyl phosphatidylethanolamine lipase [5–7]. Human ABHD5, also known as Comparative Gene Identification 58 was found to be the causative gene of human Chanarin Dorfman Syndrome, also known as the neutral lipid storage disease (NLSD) [8,9]. ABHD6 and ABHD12 were identified as 2-arachidonoylglycerol (AG) hydrolase which plays a key role in neurotransmission [10–14]. Later, ABHD12 was also found to be linked with an autosomal recessive genetic disorder called Usher Syndrome 3 [15]. Human lymphocyte antigen B-associated transcript 5 (BAT5), also known as ABHD16A was shown hydrolysing medium and long-chain unsaturated monoacylglycerols (MAGs).

Human ABHD2, previously known as lung α/β hydrolase 2 (LABH2), is one among the αβ hydrolase superfamily [16].

Abbreviations: ABHD, αβ hydrolase domain; MDS, molecular dynamic simulation; pNP, p-nitrophenyl; pNFB, pNP butyrate; pNPP, pNP palmitate; pNPA, pNP acetate; RMSF, root mean square fluctuation; TAG, triacylglycerol; w/v, weight/volume; WT, wild type; YNB, yeast nitrogen base.

1 To whom correspondence should be addressed (email jksingh@kluniversity.in).
Reduction in the number of alveolar type II cells and unusual accumulation of macrophages in the lungs was seen in aged mice by global deletion of ABHD2 [17]. In addition to its role in the lung, ABHD2 plays a significant role in macrophage infiltration to atherosclerotic lesions [18]. Collectively, ABHD2 seems to play an important role in chronic diseases, i.e. atherosclerosis and emphysema involving monocyte/macrophage recruitment. However, this putative lipase has not been studied in any detail and there are no experimental data to confirm the functionality, evolutionary relationship, substrate specificity and the role of this protein in lipid breakdown. In the present study, we report the triacylglycerol (TAG) lipase and ester hydrolase activities of human α β hydrolase2 (ABHD2).

EXPERIMENTAL

Strains and growth conditions
Yeast strains used in the present study are Saccharomyces cerevisiae BY4741 (WT), ABHD2 overexpressed in WT (OE) and only vector pYES2/CT cloned in WT (v). WT cells were grown either in YPD medium containing 1% yeast extract, 2% peptone and 2% dextrose weight/volume (w/v) or synthetic minimal medium (SC + Ura) containing 0.67% yeast nitrogen base (YNB), supplemented with the complete supplement mixture 0.192% appropriate amino acids without uracil, 2% dextrose and 0.015% uracil (w/v). Recombinant yeast strains OE and V were cultured in synthetic minimal medium without uracil (SC-Ura) containing 0.67% YNB, supplemented with the complete supplement mixture 0.19% appropriate amino acids without uracil and 2% dextrose. Induction was done in SC-Ura media with 2% raffinose and 3xYP medium with 6% galactose [19]. All cells were cultured in liquid media at 30°C with shaking at 180 rpm.

Phylogenetic analysis
Molecular phylogenetic analysis was performed with the 21 human ABHD family proteins retrieved from NCBI along with orthologous sequences of mouse, rat, Drosophila melanogaster and Arabidopsis thaliana. The tree was developed by maximum likelihood method using MEGA (version 6.0) [20]. Bootstrap values were determined from 1000 trials and the phylogenetic tree with the highest log likelihood is shown. The tree was drawn to scale and the analysis involved a total of 25 protein sequences.

Cloning and expression of recombinant ABHD2
ABHD2 was cloned into pYES2/CT vector and transformed into DH5α cells. Only vector and pYES2/CT along with the construct were further transformed into WT individually by using the Frozen-EZ Yeast Transformation kit (Zymo Research) following the manufacturer protocol. Expression of the recombinant ABHD2 in WT was performed as per Gelperin et al. [19] and purified by Ni-NTA agarose (Qiagen) column.

Esterase activity using p-nitrophenyl esters as substrates
Esterase activity was assayed with p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB) as mentioned by Ploier et al. [21]. Similarly, for p-nitrophenyl palmitate (pNPP) substrate assay was conducted as per Kanwar et al. [22]. Different concentrations of substrates (2–20 mM) were considered for analysis of enzyme kinetics with all the three substrates studied. The purified recombinant ABHD2 enzyme of 3.34 μg was used for esterase activity against pNPA, pNPB and 33.40 μg with pNPP substrates respectively. Hydrolytic activity was checked at different pH 4.5, 5.5 (sodium acetate buffer), 6.5, 7.5 (sodium phosphate buffer), 8.5 and 9.5 (Tris/HCl buffer) with all the substrates. The esterase activity was also monitored at different temperatures, including 4, 30, 45, 60, 75 and 90°C. All the assays were performed in triplicates and mean values were recorded. Michaelis–Menten kinetics was analysed using Graph Pad Prism version 5.

Tag lipase assay
TAG lipase assay was assayed using Lipase activity assay kit (K722-100; Biovision) following the manufacturer protocol. In brief, lipase hydrolyses a triacylglycerol substrate to form glycerol which is measured enzymatically at 570 nm, by monitoring a linked change in the OxiRed probe absorbance [23]. The lipase assay was carried out with 3.34 μg of purified enzyme at pH 8.5 and incubated at 37°C.

Homology modelling
The query ABHD2 sequence was retracted from the Uniprot resource (P08910) [24]. Homology modelling was carried using the Prime module of the Schrödinger suite [25]. The template for the model building was searched using the blast search bundled within the software. The secondary structure was predicted using the SSpro module. Following the ClustalW protocol, both the target and the template were aligned and finally the model was built omitting the inbuilt ligands if any. The stereo chemical quality of the model was assessed by using PROCHECK [26].

Molecular dynamic simulation studies
The model was further refined using the Desmond molecular dynamic simulations (MDS) [27]. With system builder of the Desmond module, the model was incorporated in an orthorhombic periodic boundary box and was solvated using the SPC water model. This was neutralized with 4Na+ ions based on the total charge of the model and to this system 0.15 M salt was added. This was simulated using the dynamic simulation step of the Desmond module. The simulations were carried using NPT ensemble, at 300 K temperature, one atmospheric pressure and finally the entire system was relaxed using the default protocol. This system was carried for 100 ns simulation time period and the trajectory obtained from this simulation run was analysed.
**Ligand preparation**

The structures for the molecules were retrieved from Pubchem database [28] and were prepared using LigPrep tool [29] of Schrödinger suite with OPLS_2005 as a force field. The ionization states of these molecules were generated at pH 7.0 ± 2.0 using Epik module and 32 possible stereoisomers generation per ligand was selected.

**Docking studies**

The refined model obtained from the simulation studies was further prepared using the protein preparation wizard [30] by addition of hydrogens and bond orders assignment. The model was then optimized and minimized using force field OPLS_2005 with RMSD of 0.30 Å (1 Å = 0.1 nm). A grid box was generated around the important amino acids reported previously using the receptor grid generation protocol of the Glide module [31]. The prepared protein and molecules were docked covalently using the covalent docking protocol of the glide module.

**In silico mutational analysis**

The model was point mutated with Alanine replacing the Ser^{207} of the catalytic triad and the mutated model was docked with selected pNP substrates under the similar experimental conditions opted above.

**RESULTS AND DISCUSSION**

Variations in lipid metabolism have been increasingly listed as one of the characteristic features of cancer cells. DNA-microarray data from the ONCOMINE database indicated differential expression of lipases and other proteins related to the α/β hydrolase family in various tumours [32]. In addition, the multiple data sets obtained from breast cancer in comparison with normal cells also indicated high expression of human ABHD2 gene in breast and lung cancers [33–35]. This prompted us to check the functionality of human ABHD2 protein. *In silico* sequence analysis highlighted only the putative functional role but the substrate specificities of the enzyme are not yet clearly depicted. Present study identified human ABHD2 as both TAG lipase and ester hydrolase based on experimental data.

**Domain structure**

*In silico* sequence analysis revealed human ABHD2 (P08910), as a protein of 425 amino acids containing an α/β hydrolase domain ranging from 1 to 425 amino acids and belonging to α/β hydrolase superfamily. The conserved GXSXG sequence motif is found between 205 and 209 amino acids (Figure 1A) which is the general substrate binding site of lipase and ester hydrolase enzymes. Active sites identified are serine (S^{207}), aspartic acid (D^{145}) and histidine (H^{378}) (Figure 1A). Another conserved motif HXXXXD sequence is found in the N-terminal region, ranging from 120 to 125 amino acids (Figure 1A). A trans-membrane region is also found in the protein sequence at the N-terminal spanning 10–30 amino acids (L^{10}PAFVDGKLAAGAVLYV^{30}) as predicted by DAS-TM filter server and also identified as a type II membrane protein by Innovagen peptide property calculator software with poor water solubility hydrophathy plot.

**Sequence homology and phylogenetic analysis**

Sequence comparison of human ABHD2 (gi: 23397661), with annotated databases revealed sequence similarity with *D. melanogaster* CG3488, mouse ABHD2, rat ABHD2, *A. thaliana* ABHD2 and human ABHD family members. The phylogenetic tree diverged into three groups (Figure 1B) containing, Group
Figure 2  Ester hydrolase activity of ABHD2: kinetics, pH and temperature optima with pNP substrates

(A) Highlights the optimum pH value showing maximum enzyme activity. (B) Represents the ABHD2 activity at different temperatures. (C) Purified ABHD2 enzyme of 0.67 ± 0.06 mg/ml concentration was used for the analysis of esterase activity using pNPA, pNPB and pNPP as substrates. Data were analysed using graph pad prism 5. Data are mean values from three independent experiments with respective S.D. values.

TAG lipase activity
To determine the hydrolytic activity, human ABHD2 gene was overexpressed in S. cerevisiae WT cells and the recombinant protein was purified to perform enzymatic assays. For this purpose, full-length protein of 51.7 kDa was expressed with a C-terminal 6 × His-tag using pYES2/CT vector and was successfully purified from whole cell extracts by Ni-NTA column. This affinity purified recombinant hABHD2 protein fraction showed TAG lipase activity of 1.14 ± 0.11 μmol/s·mg of protein against controls.

Esterase assay confirms hydrolytic activity
The purified recombinant ABHD2 enzyme was used for esterase activity against pNPA, pNPB and pNPP as substrates. Hydrolytic activity was screened at different pH and was found to be optimum at pH 8.5 for pNPP and at pH 7.5 for both pNPA and pNPB substrates respectively (Figure 2A). The esterase activity was also monitored at different temperatures and was found optimum at 45°C for pNPP (Figure 2B) whereas pNPA and pNPB substrates showed optimum activity at 30°C.

ABHD2 cleaved, pNPA with a $K_m$ of 12.40 ± 1.02 mM, $V_{max}$ of 2.69 ± 0.15 μmol/s·mg and $k_{cat}/K_m$ of 11.23 ± 1.22 M$^{-1}$·s$^{-1}$, pNPB with a $K_m$ of 11.76 ± 1.15 mM, $V_{max}$ of 3.71 ± 0.36 μmol/s·mg with $k_{cat}/K_m$ of 16.33 ± 1.98 M$^{-1}$·s$^{-1}$ and pNPP with a $K_m$ of 17.66 ± 2.54 mM, $V_{max}$ of 1.27 ± 0.12 μmol/s·mg with $k_{cat}/K_m$ of 3.72 ± 0.95 M$^{-1}$·s$^{-1}$ (Figure 2C). pNPP is not very water soluble and likely affects the apparent $V_{max}$, but it is found to be a substrate when compared with control data. Control assay with only pYES2/CT vector alone overexpressed and purified fractions showed no activity. All together, the present study highlights the TAG lipase activity of ABHD2 along with both long and short chain esterase activities against pNP palmitate, butyrate and acetate substrates respectively.

Homology modelling
A 3D model was required to perform the binding studies, for which we opted prime modelling tool of the Schrodinger suite. Based on the BLAST results, 1BRO of Streptomyces aureofaciens with a resolution of 2.05 Å was chosen as a template to build the structure. This protein showed 24% identity and 46% positives against our query sequence, which was the top and best among the BLAST hits obtained. The background for
ABHD2 is a TAG lipase and ester hydrolase

considering this particular PDB file apart from the above points was it contains \(\alpha/\beta\) hydrolase domain and also the important catalytic triad. The model was built (Figure 3A) based on this template and was further analysed using Ramachandran plot. 81.5 % of residues were reported to be in the most favoured regions, 15.8 % residues in additionally allowed regions (Figure 3B), 1.5 % in generously allowed regions and remaining 1.2 % only in the disallowed regions of the Ramachandran plot. Based on this result, the model was predicted as the best one and was further refined using the MDS studies.

The model was further refined and analysed based on the RMSD and RMSF of the trajectory obtained after the 100 ns simulation run. Deviations in the model were reported between 1.5 and 5.2 Å during the simulation run period. During the initial run time of simulations, the model showed huge deviations from 1.5 to 5.2 Å up to 30 ns (Figure 3C). From there, the deviations showed small declination from 5.2 Å to below 5.0 Å and further very small inclinations and declinations were reported with a steady state approximately 5.0 Å. The deviations in the initial stages were mainly due to the presence of a number of loops in the protein structure. The movement of amino acids of the modelled protein was analysed using the RMSF plot (Figure 3D). High fluctuations were reported above 5.0 Å between 100–110 and 240–250 amino acids of the model. Other residues between 140–155 and 250–265 displayed fluctuations within 4.0–5.0 Å. The remaining amino acids were reported below 3.0 Å. The major amino acids which were fluctuating rapidly during the simulations run time were present in the end loop region and attained stability by the end of dynamics time period. The fluctuation range in the modelled protein was reported approximately 0.6–5.8 Å.

**Ligand docking studies**

The frames obtained from the trajectory were divided into clusters based on their energies. The cluster, which was displaying the minimum energy was continued for the binding studies. During the homology modelling, the model was built without using the
Covalent bond formation between the ABHD2 model with (A) pNPA; (B) pNPB and (C) pNPP highlighting the interactions with specific amino acids.

Figure 4 ABHD2 docking interactions with pNP substrates

Covalent bond formation between the ABHD2 model with (A) pNPA; (B) pNPB and (C) pNPP highlighting the interactions with specific amino acids.

docked into the receptor active pocket i.e. catalytic triad. Both acetate and butyrate molecules produced covalent bond with the serine of the catalytic triad which was the key residue in performing the hydrolase activity. The developed ABHD2 model and the acetate ligand shared covalent bond between the oxygen atom of serine and carbon atom of acetate with a G-score of $-3.21$ (Figure 4A). The ABHD2–butyrate complex also produced covalent bond sharing electrons between the oxygen and carbon atoms as depicted in Figure 4(B). This complex showed G-score...
of ~3.63 which was slightly higher than the ABHD2–acetate complex. This is in agreement with the in vitro activity observed with the same substrates. Whereas in the ABHD2–palmitate complex, instead of the covalent bond, one hydrogen bond and one pi–pi stacking were observed (Figure 4C). There was no interaction between palmitate and any of the important residues of the catalytic triad because the fitting of the ligand was away from the predicted pocket. The hydrogen bond was formed with the residue Asn257 and the stacking was with Tyr114 with a G-score of ~3.50.

Further, the model was cross validated with point mutated ABHD2. The important residue of the active site Ser207 was replaced with Alanine. The mutated model showed no interactions with any of the chosen substrates when covalently docked (Supplementary Figure S1). This result highlights the role of Ser207 in forming covalent bonding with the substrates and its importance in the activity of lipases with conserved GXSXG motif. These observations correlate with the in vitro mutational studies of lipases, MGAT of Arachis hypogaea [37] Cvl17 [38] LpH1 [39], ROG1 [40] of S. cerevisiae which showed no or marginal activity when serine of GXSXG motif was mutated with alanine.

CONCLUSION

In conclusion, the human ABHD2 protein sequence shows an α/β hydrolase domain with conserved GXSXG lipase motif. To study the role of this conserved GXSXG domain, ABHD2 was cloned, overexpressed and the recombinant protein was used for in vitro enzyme assays. The results obtained with His-tag purified recombinant ABHD2 protein clearly displayed both TAG lipase and ester hydrolase activities. This functionality justifies the increase in expression of human ABHD2 gene in breast and lung cancers, supporting the necessary energy by the breakdown of lipids for the accelerated proliferation of cancer cells. Thereby, it could also serve as a selective and potential target for combinatorial cancer chemotherapy.

AUTHOR CONTRIBUTION

Naresh Kumar M. and Bondili J.S. conceived, designed the study and wrote the paper. Naresh Kumar M. and Chandra Sekhar B. designed, performed and analyzed the experiments. Thunuguntla V.B.S.C. and Swapna Guntupalli characterized enzyme activity. Thunuguntla V.B.S.C. and Veeramachaneni G.K. performed in silico studies. All authors analyzed the results.

ACKNOWLEDGEMENTS

We thank Prof. Ram Rajasekharan CSIR-CFTRI, Dr D.K. Venkata Rao, CSIR-CIMP for their kind support and help.

FUNDING

This work was supported by Council of Scientific & Industrial Research (CSIR), Ministry of Science and Technology, Government of India with CSIR-Senior Research Fellowship (SRF) [grant number 09/1068(0001)/EMR1/2011 (to N.K.M.]); and the University Grants Commission (UGC), Government of India, withUGC-Research Award [grant number F.30.1/2013 (SA-II)/RA-2012-14-GE-ANP-1237 (to B.J.S.)).

REFERENCES


© 2016 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution Licence 4.0 (CC BY).

Received 7 February 2016/16 May 2016; accepted 31 May 2016
Accepted Manuscript online 31 May 2016, doi 10.1042/BSR20160033