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Actin binding LIM protein 3 (abLIM3)

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Abstract. LIM domain proteins were demonstrated to play key roles in various biological processes such as embryonic development, cell lineage determination, and cancer differentiation. Actin binding LIM protein 1 (abLIM1) was reported to be localized in a genomic region often deleted in human cancers and suggested to be involved in axon guidance. Recently, existence of a second family member was reported, actin binding LIM protein 2. By means of computational biology and comparative genomics, we now characterized an additional, third member of the actin binding LIM protein subgroup, actin binding LIM protein 3 (abLIM3). The human mRNA sequence was previously annotated as differentially regulated in hepatoblastoma compared to normal livers. Conservation of key structural features of abLIM1 and abLIM2, four LIM domains and a VHD domain, suggested comparable biological function of abLIM3 as a linker between actin cytoskeleton and cell signaling pathways. AbLIM3 was found to be conserved in vertebrates, as orthologous sequences were characterized for mouse, fish, and frog. In addition, we report the existence of abLIM2 orthologs in fish and frog, suggesting a similar degree of evolutionary conservation. The intracellular localization of the abLIM3 protein was predicted to be nuclear by means of Reinhardt's neural network and the k-nearest neighbor algorithm. The corresponding *abLIM3* gene was localized to chromosome 5q32 and spanned 119 kb, organized in 24 exons. An RT-PCR based expression profile available from the human unidentified gene-encoded (HUGE) database demonstrated highest expression for *abLIM3* in heart, lung, liver, and brain/cerebellum accompanied by lower expression in multiple other tissues. Furthermore, *abLIM3* was expressed in fetal liver, CNS, and spinal cord.

Introduction

Over the past 15 years LIM domain proteins were demonstrated to play key roles in various biological processes such as embryonic development, cell lineage determination, and cancer differentiation. An integral part of this protein family is the LIM domain, a consensus sequence of 50-60 amino acids, first identified in three homeodomain proteins Lin-11, Islet-1 and Mec-3. The LIM domain was demonstrated to be a double zinc finger structure promoting protein-protein interactions, the main way of function of LIM domain proteins. Thus far, no single binding motif has been characterized as a common target for LIM domains (1). In addition to their role in basic biological processes, LIM domain proteins play important roles in different disease processes, among them cancer development. LIM domain only (LMO) proteins are reported to play an important role in the development of acute leukemia (2). LMO4 was shown to be involved in breast cancer differentiation (3). FHL2 was recently reported to induce ß-catenin, one of the central proteins within the Wnt signaling pathway, demonstrated to play an integral role in the development of a wide variety of malignancies, such as colorectal cancer and hepatocellular carcinoma (4). Actin binding LIM protein (abLIM1) was originally identified in homology to Dematin and suggested to be a possible bridging molecule between the actin-based cytoskeleton and signaling pathways (5). Subsequently, the corresponding gene was found to be localized within a genomic region of frequent loss of heterozygosity and thus speculated to be a candidate tumor suppressor gene (6). However, since the main focus of investigating abLIM function has been on its role in axon path finding, which has been investigated in organisms as diverse as worm and mouse (7-9). Recently, Klimov *et al* demonstrated the existence of a second family member, actin binding LIM protein 2. They demonstrated comparable structural features, four LIM domains and a VHD domain and demonstrated conservation in mammals (10).

Due to the combined efforts of the human genome project, the mouse genome group, an many other sequencing efforts the human, mouse as well as a wide variety of additional genomic sequences are now publicly available. While most chromosomes of the human genome are completely assembled, assembly on some genomic fragments is currently still underway and most chromosomal locations are still undergoing preliminary bioinformatics analysis. In addition, large parts of the genome have yet to be analyzed in detail for their genomic content, and many genes, while completely sequenced, have not yet been identified or characterized.

In an effort to screen the available human genomic information for new genes potentially involved in cancer differentiation, we have characterized an additional member of the actin binding LIM subset of LIM domain proteins, actin binding LIM protein 3 (abLIM3).

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Materials and methods

Genomic database. All database searches were performed on the latest available releases of the genomic NCBI human, mouse, zebrafish, and frog databases, NCBI 35, NCBI m33, WTSI Zv4, and JGI3 respectively (11-13).

Identification of the human abLIM3 protein and corresponding genes. The human abLIM3 protein was identified by a BLAST search (14,15) comparing the human abLIM1 protein sequence (isoform a, accession no. NP_002304) against the non-redundant database. The new protein sequence (accession no. NP_055760) initially retrieved was subsequently re-BLASTed against the protein database, in order to identify additional sequence entries or short protein fragments. The abLIM3 protein sequence was finally BLASTed against the translated protein database to identify the corresponding human *abLIM3* mRNA NM_014945.

Identification of abLIM3 orthologs in other species. Orthologs corresponding to the human abLIM3 were identified by BLAST search (15). The human abLIM3 was used as query sequence and BLASTed against the est_mouse, est_others, and other non-redundant databases.

Exon-intron structure and chromosomal localization. Exon-intron structure and chromosomal localization was identified by BLASTing the human *abLIM3* mRNA sequence (NM_014945) against the human genome draft sequences (htgs). In addition, exon-intron structures were annotated at NCBI Entrez Gene (16) and Ensembl (17).

Analysis of the human 5q32 locus. Linkages of the human 5q32 locus to human diseases were obtained from the OMIM (18) and Pubmed (19) databases.

Analysis of protein structure and phylogenesis. Alignment of human abLIM sequences were performed using the ClustalW algorithm at EBI (20) and the GeneBee web server (21). Protein structures and domains were analyzed using two independent algorithms, Pfam (22) and NCBI Conserved Domain Search (23).

Prediction of subcellular localization. The subcellular localization of abLIM3 was predicted using two independent algorithms, the neural network constructed by Reinhardt and Habbard (24), as well as the k-nearest neighbor algorithm (25).

In silico expression analysis. A complex expression profile was predicted using the GeneNote annotations of the Weizmann Institute of Science (26). In addition, GenBank (11) was searched for *abLIM3* ESTs and an RT-PCR based expression profile of *abLIM3* was available from the Database of Human Unidentified Gene-Encoded Large Proteins Analyzed (HUGE) (27).

Results

Identification of the human abLIM3 protein sequence and corresponding gene. Searching for new homologous members of the human abLIM family, we BLASTed (14,15) the human abLIM1 sequence (NP_002304) against the non-redundant protein database. Thus, we were able to match a considerably similar, yet uncharacterized protein sequence. This sequence was previously deposited GenBank (11) by large throughput approaches to identify hepatoma related genes (28), to generate more than 15,000 full-length human and mouse cDNA sequences (30), and to predict the coding sequences of unidentified human genes (29) under the accession no. NP_055760. Due to the newly characterized protein's structural features and homology to the human abLIM1 and abLIM2 sequences, the protein was annotated as actin binding LIM protein 3, abLIM3. The abLIM3 protein sequence was BLASTed against the translated protein database to identify the corresponding human *abLIM3* mRNA sequence NM_014945. Aligning the human mRNA sequence with the human genome draft sequence, the corresponding *abLIM3* gene was localized to chromosome 5q32.

Identification of abLIM3 orthologs in other species. Orthologs corresponding to the human abLIM3 sequence were identified by BLAST search (14,15). The human abLIM3 (NP_055760) was used as a query sequence and BLASTed against the est_mouse, est_others databases, and other available non-redundant databases.

Orthologs of abLIM3 were identified in mouse (NP_941051, predicted, 682 aa), zebrafish (ENSDARP00000052594, 686 aa), and frog (ENSXETP00000045032, 663 aa). ClustalW alignment demonstrated a 98% identity between the human and mouse protein sequences, a 73% identity between the human and fish protein sequences, and a 79% identity between the human and frog sequences. No orthologous sequence were found in *Drosophila* or *C. elegans*. In summary, the abLIM3 protein was found to be conserved in vertebrates.

As abLIM1 and abLIM3 both demonstrated conservation in non-mammalian species, we also searched the available genomic databases for abLIM2 ortholog sequences. In addition to the recently published mammalian abLIM2 orthologs in human, mouse, and rat, we demonstrated abLIM2 orthologous sequences in zebrafish (ENSDARP00000046045, 551 aa) and frog (ENSXETP00000025375, 586 aa). Alignment of human, zebrafish, and frog protein sequences were performed using the ClustalW algorithm (20). These alignments demonstrated an identity between the human and zebrafish and human and frog sequences of 57% and 70%, respectively. Currently, no abLIM2 orthologous sequences were found in fly or worm. Thus, like abLIM3, the abLIM2 protein must be assumed to be conserved in vertebrates.

Exon-intron structure and chromosomal localization of the human and mouse abLIM3 genes. Exon-intron structure and chromosomal localization were identified by BLASTing the human *abLIM3* mRNA sequence (NM_014945) against the human genome draft sequences (htgs) of chromosome 5q32, annotated at Ensembl/EBI (17) and Entrez Gene/NCBI (16).

The human abLIM3 gene spanning approximately 119 kb was organized into 24 exons. With exemption of exon 24, containing a large 3' UTR of 2,032 bp, exons were rather small ranging between 8 bp and 184 bp. Exon 24 contained 2,164 bp. Comparing the cDNA sequence and the human protein sequence, the cDNA was demonstrated to contain a

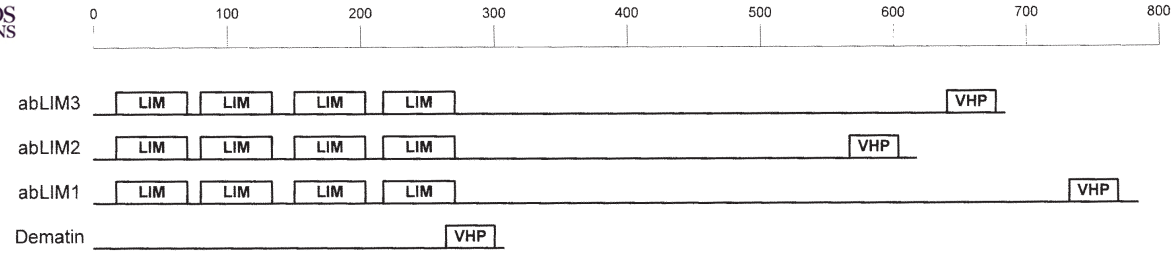

Figure 1. Schematic alignment of the human abLIM family and Dematin.

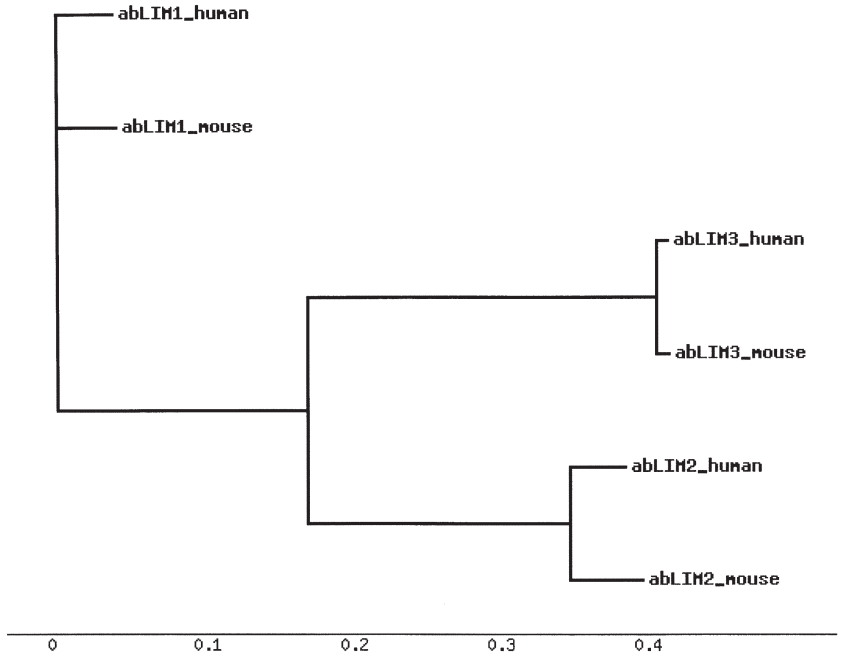

Figure 2. Phylogenetic analysis of the human and mouse abLIM protein sequences.

5' UTR of 172 bp, exon 1 was untranslated and the translation start was localized in exon 2. Intron sizes were predicted to be between 354 bp and 41,343 bp.

The mouse *abLIM3* gene spanning approximately 110 kb was organized into 24 exons, as well. Again, with exeption of exon 24, containing a large 3' UTR of 1,903 bp, exons were rather small ranging between 8 bp and 184 bp. Exon 24 contained 2,017 bp. Comparing the cDNA sequence and the human protein sequence, the cDNA was demonstrated to contain a 5' UTR of 235 bp, exon 1 was untranslated and the translation start was localized in exon 2. Intron sizes were predicted to be between 347 bp and 39 kb.

Genomic locus 5q32. Since *abLIM1* has been found to be localized in a genomic region often deleted in human cancers, the genomic locus containing *abLIM3*, 5q32, was searched for linkage to human disease especially cancer differentiation. However, searching the OMIM database (18), no relevant linkage to cancer disease could be identified.

Analysis of abLIM3 protein structure. In order to identify conserved protein domains, the human abLIM3 protein sequence was analyzed using two independent algorithms, Pfam (22) and NCBI Conserved Domain Search (23). For human abLIM3, the Pfam database predicted LIM domains between amino acids 23 and 79, 82 and 139, 151 and 207, as well as 210 and 267. A VHD domain was predicted between amino acids 648 and 683. The NCBI Conserved Domain Search confirmed these results identifying LIM domains between amino acids 23 and 73, 81 and 133, 150 and 201, and 209 and 247. The VHD domain was predicted between amino acids 648 and 683 (Fig. 1).

These structural features were conserved in mouse. The Pfam database identified LIM domains between amino acids 23 and 79, 82 and 139, 151 and 207, and 210 and 267 as well as a VHD domain between amino acids 647 and 682 of the mouse abLIM3 sequence. Again, the NCBI Conserved Domain Search confirmed these results, identifying LIM domains between amino acids 23 and 73, 81 and 133, 150 and 201, and 209 and 247. The VHD domain was predicted between amino acids 648 and 682. Searching the fish and frog abLIM3 protein sequences for conserved domains, using the same bioinformatics algorithms, demonstrated a conservation of these domains in both non-mammalian species.

Phylogenetic analysis comparing the human and mouse protein sequences of the abLIM group demonstrated a closer relationship between the newly abLIM3 and abLIM2 and more distant homology to abLIM1 (Fig. 2).

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abLIM1_human      HFHVPDQGIN--IYRKPPIYKQHAALAAQSKS---SEDIIKFSKFPAAQAPDPSETPKIE 565
abLIM1_mouse      HFHVPDQGIN--IYRKPPIYKQHAALAAQSKA---SEDIIKFSKFPAAQAPDPNEIPKIE 649

abLIM2_mouse      HFHVPDTGVKDNIYRKPPIYKQHAARRL---------DVEDSSFDQDSRKK--------- 461
abLIM2_rat        HFHVPDTGVKDNIYRKPPIYKQHAARRL---------DVEDSSFDQDSRKK--------- 461
abLIM2_human      HFHVPDTGVKDNIYRKPPIYRQHAARRS---------DGEDGSLDQDNRKK--------- 460
abLIM2_frog       LFSSIDSGVKDNIYRKPPIYKQHGIKKVFSI------KSESISTHFSVQVK--------- 440
abLIM2_fish       HFHIPETKVKDNIYRKPPIYKQHG----------------------TVQSP--------- 417

abLIM3_human      HFHIPA-GDSN-IYRKPPIYKRHG-DLSTATKSKTSEDISQTSKYSPIYSPDPYYASESE 486
abLIM3_mouse      HFHIPA-GESN-IYRKPPIYKRHG-DLSTATKSKTSEDISQASKYSPAYSPDPYYASESE 485
abLIM3_fish       HFHLPATGEPN-IYRKPPIYKRHDNHCNPATKSKTSEDIVQSSKFPSAYSPEHYQHSESD 471
                   *          ********::*
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Figure 3. Sequence alignment of the abLIM protein sequences demonstrated a highly conserved region of 11 amino acids between amino acid 442 and 452 of the abLIM3 sequence with currently unknown function.

Protein localization. Protein localization was analyzed according to the neural network of Reinhardt and Hubbard and the k-nearest neighbor algorithm for assessing the probability of the proteins localization. The neural network of Reinhardt and Hubbard (24) predicted a nuclear localization for abLIM3 with a reliability of 94.1%. This prediction was supported by the k-nearest neighbor algorithm (25) also predicting a nuclear localization for abLIM3 with a reliability level of 95.7%.

Expression analysis. The *abLIM3* sequence had also been annotated at GenBank as KIAA0843. For this sequence an initial, randomly performed, RT-PCR based expression profile was available from the Database of Human Unidentified Gene-Encoded Large Proteins Analyzed (HUGE) (27). According to these results, *abLIM3* was expressed at considerable levels in heart, lung, liver, and brain/cerebellum accompanied by a rather broad expression profile in many other tissues. Lowest/missing expression was demonstrated in pancreas. Finally, *abLIM3* was also expressed in fetal liver, brain, and spinal cord, suggesting a role during embryonic development.

Discussion

LIM domain proteins were demonstrated to play key roles in various biological processes such as embryonic development, cell lineage determination, and cancer differentiation. Actin binding LIM (*abLIM1*) was found to be localized within a genomic region of frequent loss of heterozygosity and thus speculated to be a candidate tumor suppressor gene (6). Recently, Klimov *et al* demonstrated the existence of a second family member, actin binding LIM protein 2, in mammals (10). In an effort to screen the available human genomic information for new genes and corresponding proteins potentially involved in cancer differentiation, we have characterized an additional member of the abLIM subset of LIM domain proteins, abLIM3, solely by means of computational biology and comparative genomics.

Demonstrating different genomic localization for the corresponding *abLIM1, abLIM2*, and *abLIM3*, on chromosomes 10q25, 4pl6-pl5 and 5q32, excluded the possibility of the *abLIM3* gene and corresponding protein being a (splice) variant of *abLIM1* or *abLIM2*.

Comparing the structural features of the newly characterized protein, abLIM3 was demonstrated to be member of the abLIM family, as the human, mouse, fish, and frog abLIM3 proteins all displayed four LIM domains and a villin head piece (VHD) domain, comparable to abLIM1 and abLIM2. The VHD headpiece had primarily been classified as an F-actin-binding domain. However, it has been shown that not all headpiece domains are intrinsically F-actin-binding motifs. AbLIM1 was originally identified due to the common VHD domain and its similarity to the Dematin sequence. However, in contrast to Dematin, all three abLIM proteins displayed four LIM domains, known to be responsible for protein-protein interaction (Fig. 1). These LIM domains were located on the N-terminal end of the protein sequence and distinguish the abLIM proteins from the Dematin sequence. Thus, it seemed reasonable to regard the abLIM proteins as a separate subset of proteins, distinct from Dematin or other VHD domain containing proteins.

Since abLIM1 and abLIM2 have been demonstrated to bind actin, a similar function must also be assumed for the homologous abLIM3 protein. Since LIM domains are known to serve as protein-protein interaction domains, a function of abLIM3 as a bridging molecule between signaling pathways and actin cytoskeleton must be assumed.

Aligning the human abLIM sequences and their orthologous sequences from other species, regions of higher similarity were found at the N-terminus where the LIM domains are located as well as at the C-terminus where the VHD domain was identified. Interestingly, a highly conserved region of 11 amino acids was identified between amino acid 442 and 452 of the abLIM3 sequence with perfect match in all sequences over the first 8 amino acids (Fig. 3). This high conservation suggests a functionally highly important part of the protein sequence. However, searching Pfam and NCBI Conserved Domain Search as well as BLASTing these 11 amino acids against the complete non-redundant NCBI database did not reveal any matches to any currently known structures or domains. Therefore, the function of this short domain currently remains unclear.

By phylogenetic analysis, closer relationship between the newly characterized abLIM3 and abLIM2 was demonstrated (Fig. 2). All three abLIM proteins demonstrated considerable conservation throughout evolution. A conservation in worm, as demonstrated for abLIM1 (UNC-115) (7) could not be demonstrated for abLIM2 or abLIM3. However, the abLIM3 protein was demonstrated to be conserved in vertebrates as orthologs were found in mouse, fish and frog. As Klimov *et al* had described only mammalian abLIM2 sequences (10), we

ched the non-mammalian databases for conserved orthologs and were able to report the existence of abLIM2 orthologs in fish and frog. Therefore, the conservation of abLIM2 in vertebrates was comparable to abLIM3.

Since Kim *et al* reported *abLIM1* to be located within a genomic region of frequent loss of heterozygosity and thus speculated abLIM1 to be a candidate tumor suppressor gene (6) we searched the OMIM database for a possibly role *abLIM3* containing loci in tumor differentiation. However, no relevant linkage had been annotated for this region.

Finally, it has to be acknowledged that the mRNA record of the human and mouse sequences is supported by experimental evidence of 170 human and 140 mouse ESTs as well as mRNA sequences reported by three different groups. However, the coding sequence of abLIM3 was still predicted but demonstrated all conserved domains as well as a perfect match to the highly conserved area between amino acids 442 and 452.

Analysis of these ESTs and the RT-PCR based expression profile, available from the human unidentified gene-encoded (HUGE) (27) database, demonstrated highest expression for abLIM3 in heart, lung, liver, and brain/cerebellum accompanied by lower expression in multiple other tissues. Furthermore, abLIM3 was expressed in fetal liver, CNS, and spinal cord. With this high expression in adult and fetal liver, the expression profile of the *abLIM3* gene supports a suggested role of abLIM3 in hepatoma development.

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