A New Functional Motif in Hox Domain-containing Ceramide Synthases

IDENTIFICATION OF A NOVEL REGION FLANKING THE Hox AND TLC DOMAINS ESSENTIAL FOR ACTIVITY

Adi Mesika1, Shifra Ben-Dor1, Elad L. Laviad1, and Anthony H. Futerman1

From the 1Departments of Biological Chemistry and 2Biological Services, Weizmann Institute of Science, Rehovot 76100, Israel

Ceramide is synthesized in mammals by a family of ceramide synthases (CerS) each of which uses a relatively restricted set of fatty acyl-CoAs for N-acylation of the sphingoid long chain base (Pewzner-Jung, Y., Ben-Dor, S., and Futerman, A. H. (2006) J. Biol. Chem. 281, 25001–25005). CerS are characterized by two functional domains, the Tram-Lag-CLN8 (TLC) domain and the homeobox (Hox) domain, which is found in all mammalian CerS except CerS1. We now demonstrate that the majority of the Hox domain is not required for CerS activity since its deletion in CerS5 does not affect activity. Subsequently, we define a highly conserved new motif of 12 amino acid residues that flanks the Hox and TLC domains but is not part of the TLC domain, which is essential for CerS5 and CerS6 activity. Two positively charged residues in this domain, one of which is conserved in all putative CerS in all organisms, are essential for activity since site-directed mutagenesis of either (Lys-134 and Lys-140 in CerS5) results in an ~50% loss of activity, whereas mutation of both leads to a complete loss of activity. Because this region is conserved across species, we propose that it plays a previously unidentified and essential role in CerS activity and can be used as a new motif to define Hox domain-containing CerS.

Most of the enzymes in the pathway of sphingolipid synthesis have recently been identified (1). These include a family of six mammalian ceramide synthase genes, originally named Lass (longevity assurance) genes but more recently named ceramide synthases (CerS)2 (2) to more accurately reflect their function. CerS consists of a sphingoid long chain base to which a fatty acid is attached via an amide bond. Each CerS utilizes a relatively restricted subset of acyl-CoAs for ceramide synthesis (3–6). The reason that mammals and other species have multiple CerS genes is not known but implies an important role for ceramides containing specific fatty acids in cell physiology.

The mammalian CerS proteins contain a number of distinguishing features. Prominent among these are two domains, (i) the Tram-Lag-CLN8 (TLC) domain, defined in a broad manner as a region of ~200 residues also found in other proteins (7) and in a narrower manner as the Lag domain, a region of ~50 amino acid residues (8), and (ii) a homeobox (Hox) domain (9), which is found in all mammalian CerS genes apart from CerS1 (2, 8) and not found in CerS from many lower organisms. Based on the presence, or not of a Hox domain, we designate CerS as (Hox)CerS (Hox domain-containing CerS) or (non-Hox)CerS (non-Hox domain-containing CerS).

Recent studies have provided evidence that the catalytic activity of CerS proteins resides in the TLC domain (10, 11). In the first of these studies histidine and aspartate residues were identified in the Lag domain of a yeast CerS homolog, Lag1p, that were essential for activity (10). In the second study eight key residues were identified in the Lag domain of mammalian CerS1, and based on this, it was suggested that the Lag motif is essential for the enzymatic activity of all Lag homologs (11). These results are consistent with both bioinformatics (7, 8) and biochemical approaches (12, 13).

In the current study we determine the role of the Hox domain in CerS activity and demonstrate that the majority of the Hox domain of CerS5 is not required for catalytic activity. However, and surprisingly, we show that a highly conserved region at the end of the Hox domain, that is, adjacent to the TLC domain but not part of the TLC domain, is essential for CerS5 and CerS6 activity. We also demonstrate that two positively charged conserved residues in this region are critical for catalytic activity and suggest that this region is a new motif that can be used to define (Hox)CerS.

EXPERIMENTAL PROCEDURES

Materials—d-erythro [4,5-3H]Sphinganine was synthesized as described (14). d-Erythro-palmitoyl-sphingosine was from Matreya (Pleasant Gap, PA). Palmitoyl-CoA was from Avanti Polar Lipids (Alabster, AL). Poly-L-lysine, defatted bovine serum albumin, phenylmethylsulfonyl fluoride, leupeptin, anti-pain, aprotonin, and an anti-FLAG antibody were from Sigma-Aldrich. Gel Mount was from Biomedia (Foster City, CA). An anti-protein disulfide isomerase antibody was from Stressgene (Victoria, BC, Canada), and an anti-hemagglutinin (HA) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).
A Functional Motif in Hox Domain-containing Ceramide Synthases

TABLE 1

| Primers used in this study | Primers used to delete the Hox domain in mouse CerS5 | Primers used for site-directed mutagenesis of mouse CerS5 | Primers used for human CerS6 |
|----------------------------|-----------------------------------------------------|-------------------------------------------------------|-----------------------------|
|                             | CerS5Δ129–135                                        | CerS5 K134R                                            | CerS6 R131K                 |
|                             | C-terminal Anti-sense                                 | Sense 5′-CTCCACAGCTTCAGGAAATTTCTCAG                     | Sense 5′-CTTCGTGGAGAATGCTTTATG |
|                             | CerS5 K134A                                          | Anti-sense 5′-CTCTGGCAAGTAGTGGACACTTTTTTATTTGGGATTCTCTT | Anti-sense 5′-CTTCGTGGAGAATGCTTTATG |
|                             | CerS5 K140R                                          | Sense 5′-CTTCGTGGAGAATGCTTTATTTGCAGGCCGAGTTCTTTTCGATGC | Sense 5′-CTTCGTGGAGAATGCTTTATG |
|                             | CerS5 K140A                                          | Anti-sense 5′-CTTCGTGGAGAATGCTTTATTTGCAGGCCGAGTTCTTTTCGATGC | Anti-sense 5′-CTTCGTGGAGAATGCTTTATG |
|                             | CerS5 K140A/K134A                                    | Anti-sense 5′-CCACATGCTTTCAGAATCGCTGGAGAGTCTCTGAGTC    | Anti-sense 5′-CTTCGTGGAGAATGCTTTATG |
|                             | CerS5 K140R/K134R                                    | Anti-sense 5′-CCACATGCTTTCAGAATCGCTGGAGAGTCTCTGAGTC    | Anti-sense 5′-CTTCGTGGAGAATGCTTTATG |

| Primers used to delete the Hox domain in mouse CerS5 | Primer A 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(A) |
|-----------------------------------------------------|-------------------------------------------------------------------------------|
| CerS5Δ129–135                                        | Primer B 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(B) |
| CerS5Δ128                                            | Primer C 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(C) |
| CerS5Δ87–128                                         | Primer D 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(D) |
| CerS5Δ36–140                                         | Primer E 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(E) |

| Primers used for deletion constructs of residues 129–140 of mouse CerS5 | Primer F 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(F) |
|-----------------------------------------------------------------------|-------------------------------------------------------------------------------|
| CerS5Δ129–135                                                         | Primer G 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(G) |
| CerS5Δ128                                                            | Primer H 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(H) |
| CerS5Δ87–128                                                         | Primer I 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(I) |
| CerS5Δ36–140                                                         | Primer J 5′-AAAAAGCTTGAGATGCTTTATTTGGGATTCTCTTCAAGGAGGAGGTGGTGTGTTTCGATGC(J) |

Horseradish peroxidase was from The Jackson Laboratory (Bar Harbor, ME). The ECL detection system was from Amersham Biosciences. Pfu polymerase was from Promega (Madison, WI) or from Stratagene (La Jolla, CA). Lipofectamine 2000 was from Invitrogen. Silica gel 60 TLC plates were from Merck. All solvents were of analytical grade and were purchased from Bio-Lab (Jerusalem, Israel).

Bioinformatics—ScanProsite (15) was run to identify all sequences in the Swiss-Prot and TrEMBL databases with a TLC domain (accession number PS50922). Those with a TLC domain were further analyzed to determine which have Hox domains (accession number PS50071). After culling sequence fragments, predicted sequences, and redundancies, a final data set of 32 proteins was obtained. Alignments were performed using ClustalW Version 1.83 (16). Logos were created using WebLogo version 2.8.2 (17). Putative CerS were determined by building phylogenetic trees of all of TLC domain-containing proteins, on which they formed a distinct branch. Due to the large number of non-Hox sequences, representative species were chosen to build the alignment and logos. SMART motif analysis (18) was performed on mouse CerS5. Analysis of the sequence of the last 12 residues of the Hox domain was performed after determining the most common residue (or residues) in each position and comparing with the 387 Hox sequences in Gehring et al. (9); data base searches were performed using Findpatterns from the GCG package (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA).

Cloning of Murine CerS5 and Human CerS6—C-terminal HA-tagged murine CerS5 cDNA was cloned into a pcDNA3 vector as described (4). Murine CerS5 deletion constructs were generated using primers with unique restriction sites (Table 1). PCR products were generated using Pfu polymerase. Site-directed mutagenesis was performed by one-step PCR using Pfu Ultra and the primers listed in Table 1.

N-terminal FLAG-tagged human CerS6 (kindly provided by Dr. Richard Kolesnick, Sloan Kettering Institute, NY) was cloned into a pCMV-Tag2B vector. Deletion constructs of human CerS6 were prepared using the primers listed in Table 1, and PCR products were cloned into pCMV-Tag2B (BamH1/EcoRI). Site-directed mutagenesis was performed by one-step PCR using Pfu Ultra and the primers listed in Table 1. The sequences of all constructs were confirmed by nucleotide sequencing.

Cell Culture and Transfection—COS-7 cells were grown on glass coverslips for morphological analyses. Human embryonic kidney (Hek) 293T cells were grown in tissue culture dishes for biochemical studies. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transfection of Hek293 cells was performed by the calcium phosphate
A Functional Motif in Hox Domain-containing Ceramide Synthases

3H-Labeled lipids were visualized by thin layer chromatography using chloroform/methanol (50:3.5, v/v) as the developing solvent, recovered from the thin layer chromatography plates by scraping the silica directly into scintillation vials, and quantified by liquid scintillation counting.

Western Blotting—Proteins were separated by SDS-PAGE, and Western blotting was performed as described (20). HA-tagged proteins were identified using a rabbit anti-HA antibody (1:2000 dilution), and FLAG-tagged proteins were identified using a mouse anti-FLAG antibody (1:10,000 dilution). Goat anti-mouse or rabbit horseradish peroxidase (1:10,000 dilution) were used as secondary antibodies. Detection was performed using the ECL detection system.

Immunolocalization—Thirty-six hours after transfection, COS cells were fixed with 3% paraformaldehyde in phosphate-buffered saline, 5% sucrose for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (10 min, room temperature). Cells were incubated with primary antibodies (mouse anti-protein disulfide isomerase, 1:400 dilution; rabbit anti-HA, 1:300 dilution) for 1 h at room temperature, Cy3-conjugated AffiniPure goat anti-mouse and Cy5-conjugated AffiniPure goat anti-rabbit secondary antibodies were diluted 1:800. After 1 h of incubation, slides were mounted in Gel Mount™ and examined by confocal laser scanning microscopy using an Olympus Fluoview FV500 imaging system. Fluorescein isothiocyanate and rhodamine fluorescence were detected using an argon and two helium-neon laser with excitation wavelengths of 488 and 543 nm, respectively. Images were acquired in sequential mode and analyzed using Fluoview 3 imaging software.

RESULTS

Sequence Analysis of the Hox Domain in CerS Genes—The Hox domain is derived from homeobox proteins, sequence-specific transcription factors important in development (9). In mammals all CerS genes apart from CerS1 have a Hox domain, but no Hox domains are found in yeast and plant CerS (2). The function of the Hox domain in (Hox)CerS is not known, but there is no evidence that the Hox domain acts as a transcription factor in (Hox)CerS genes (2).

To attempt to determine the role of the Hox domain in (Hox)CerS genes, we performed a series of bioinformatics and biochemical studies. First, TLC domain-containing proteins were identified using the Prosite domain data base (15). The entries were analyzed to determine which have a Hox domain. 32 proteins were selected for further analysis from a wide range of organisms. The Hox domains in most of these proteins were incomplete, with the N-terminal portion of the domain missing. The proteins were aligned, and the portion of the Hox domain present in all sequences was used to build a sequence logo (17) (Fig. 1). The major portion of the Hox domain (from...
residues 1–34 of the domain) is generally variable. However, conservation is much greater in the distal portion of the Hox domain in (Hox)CerS, particularly in the last 11 residues (Fig. 1). In this region the sequence is almost invariable and is enriched in positively charged amino acids. The conservation is maintained over a wide range of species, from sea squirt to man, including insects (fly and mosquito), non-mammalian vertebrates (fish and frog), and several species of mammals (supplemental Table 1).

Deletion of the Hox Domain in Mouse CerS5—To determine whether the Hox domain plays a role in the catalytic activity of mouse CerS5, we prepared a series of deletion constructs. The initial constructs were based on SMART data base predictions (18), which suggest an overlap of two residues (139–140) between the Hox and TLC domains (residues 78–140 and 139–340, respectively). In contrast, according to Prosite (15), there is no overlap between the domains (with the Hox domain designated as residues 92–136 and the TLC domain as residues 139–340), with residues 137 and 138 not designated as part of either domain. The difference between SMART and Prosite predictions is due to a less stringent definition of the Hox domain in SMART.

Based on the SMART predictions, two constructs were made, Δ82–140, which removed the entire Hox domain, and Δ87–128, which removed most of the Hox domain but left most of the highly conserved region immediately before the TLC domain intact. Surprisingly, removal of 42 amino acids (CerS5Δ87–128) from the Hox domain had no effect on CerS activity (Fig. 2). In contrast, removal of an additional 5 residues at the N-terminal end together with another 12 residues at the C-terminal end of the Hox domain (CerS5Δ82–140) led to a complete loss of activity (Fig. 2). This suggests that the majority of the Hox domain can be removed from CerS5 without loss of catalytic activity and implies that the highly conserved region immediately before the TLC domain may be required for activity.

Identification of a Region Adjacent to the TLC Domain Essential for Activity—We next tested whether this conserved region (residues 129–140) plays a role in catalytic activity. Deletion of the entire sequence (CerS5Δ129–140) led to a complete loss of activity (Fig. 3). To attempt to resolve the conflict between the SMART and Prosite predictions, we prepared two additional constructs, CerS5Δ129–135 and CerS5Δ135–140, each of which displayed ~50% activity (Fig. 3).

In wild type CerS5, a positively charged lysine residue, Lys-140, is located next to a conserved sequence, FCES, which designates the beginning of the TLC domain. Upon deletion of residues 135–140, Lys-134 becomes adjacent to the FCES sequence. To ascertain whether Lys-134 could act in a similar fashion to Lys-140 in this deletion construct, we prepared another construct in which Lys-134 was no longer adjacent to FCES (CerS5Δ136–140) due to the presence of a proline residue. This construct did not display any CerS activity (Fig. 3).
suggesting that a lysine residue must be located adjacent to FCES for catalytic activity.

To determine the role of Lys-134 and Lys-140, we mutated these residues to another positively charged residue (arginine, i.e. K134R and K140R) or to a neutral residue (alanine, i.e. K134A and K140A). Mutation of either residue to alanine led to an ~50% loss of activity, and a double mutation (K134A/K140A) led to complete loss of activity (Fig. 4). In contrast, mutation of either or both residues to arginine had essentially no effect on activity (Fig. 4). We conclude that two positively charged amino acids, residues Lys-134 and Lys-140, are required for maximal CerS activity of CerS5.

We next examined whether the loss of activity of K140A, K134A/K140A, and the CerS5Δ129−140 deletion was due to a change in $K_m$ or $V_{max}$ using sphinganine as substrate. The $K_m$ of CerS5 was 3.6 μM, with a $V_{max}$ of 215 pmol of dihydroceramide synthesized/mg/min (Fig. 5). The $V_{max}$ of K134A/K140A and CerS5Δ129−140 were close to the limit of detection and similar to mock-transfected cells (31 and 24 pmol/mg/min, respectively), and therefore, $K_m$ values could not be accurately determined. A significant reduction in the $V_{max}$ of K140A was observed (92 pmol/mg/min) compared with CerS5, with a small change in $K_m$ (1 μM) (Fig. 5). We, therefore, suggest that the loss of activity is likely due to a change in $V_{max}$ rather than a change in affinity toward sphinganine.

Because this region is highly conserved between (Hox)CerS proteins (Fig. 1), we examined whether positively charged residues are also required proximal to the TLC domain in another (Hox)CerS protein. For this purpose, we used human CerS6, which contains an arginine residue, rather than a lysine, immediately before FCES (Fig. 6). Mutation of Arg-131 to lysine (R131K) had no effect on activity, whereas 50% activity was lost upon mutation to alanine (R131A) (Fig. 6). As expected, deletion of the entire 12-residue sequence before the TLC domain resulted in complete loss of activity (Fig. 6). Thus, we conclude that the conserved positively charged residues proximal to the TLC domain in (Hox)CerS proteins are required for maximal activity.

Finally, we examined the effects of various mutations on CerS activity measured by metabolic labeling using [4,5-3H]sphinganine. The results (Fig. 7) were similar to those obtained by in vitro analysis (Figs. 2−6). [4,5-3H]Dihydroceramide synthesis was similar in CerS5Δ87−128 compared with wild type CerS5, confirming that most of the Hox domain can be removed without affecting CerS activity, whereas no increase in [4,5-3H]dihydroceramide synthesis in CerS5Δ82−
A Functional Motif in Hox Domain-containing Ceramide Synthases

SEPTEMBER 14, 2007 • VOLUME 282 • NUMBER 37
JOURNAL OF BIOLOGICAL CHEMISTRY

140 or CerS5Δ129–140-transfected cells was observed compared with mock-transfected cells. A role for the positively charged residues, Lys-134 and Lys-140 was confirmed since there was no increase in [4,5-3H]dihydroceramide synthesis upon overexpression of the double mutation, K134A/K140A (Fig. 7). All of the constructs were expressed at normal levels, and all were located to the endoplasmic reticulum (Fig. 8), demonstrating that protein mis-localization was not responsible for the loss of activity.

DISCUSSION

There are two major observations in the current study. In the first we show that most of the Hox domain is not required for the catalytic activity of CerS. This result by itself is perhaps not surprising, since one mammalian CerS, namely CerS1, and also yeast and plant CerS do not contain Hox domains and yet effectively function as CerS proteins. However, the yeast CerS, Lag1p, which is most homologous to CerS1 (2), requires another protein, Lip1, for its activity (21). No data are currently available about whether mammalian CerS1 requires additional subunits for its activity, since this enzyme has not been purified to homogeneity, but the possibility that CerS1 is regulated differently to other CerS is consistent with phylogenetic analysis, which reveals that CerS1 is on an entirely separate branch of the phylogenetic tree (2). Mammalian (Hox)CerS, at least CerS5 (20), do not require an additional subunit.

Various definitions of Hox domains exist, but the most accepted suggests that Hox domains contain 60 residues (9). In

Although we cannot formally exclude the possibility, we considered it unlikely that the mutations effect the stability of CerS due to the following. (i) There was no change in the protease sensitivity (tested using trypsin, 37 °C for 2 h) of CerS5 compared with CerS5Δ129–140. (ii) Residues 129–140 are believed to be located in a loop of the protein, according to a number of different topology prediction programs, rather than the transmembrane domains, which is a region less likely to be involved in protein folding; similar conclusions were drawn from study of the yeast CerS, Lag1p (10). (iii) Removal of 42 amino acids (CerS5Δ87–128) from the Hox domain had no effect on CerS activity (Fig. 2). (iv) We mutated Lys-134 to a charged residue, Arg, and to a neutral residue, Ala; the former resulted in essentially no loss of activity, whereas a more significant loss of activity was observed in the latter (Fig. 4B); the opposite result would be expected if the addition of a charged residue affected protein stability and/or folding. (v) No changes in protein levels and no anomalous bands were seen upon expression of any of the mutations or of the deletion construct in any of our analyses (see for instance Figs. 3B and 4B). Together with the normal localization of the mutants (Fig. 8), we, therefore, consider that a major change in protein structure and, hence, stability is highly unlikely to account for our observations.

FIGURE 6. A similar sequence upstream of the TLC domain is required for enzymatic activity in CerS6. A, sequence of human CerS6, deletion constructs, and residues altered by site-directed mutagenesis. The predicted Hox (gray box) and TLC (empty box) domains are indicated. B, homogenates (25 μg of protein) were prepared from cells overexpressing each of the constructs, and CerS activity was assayed. Results are the means ± S.D. of two independent experiments. The inset indicates levels of expression of each of the constructs, ascertained by Western blotting using an anti-HA antibody.

FIGURE 7. [3H]Dihydroceramide synthesis in CerS5 overexpressing cells. Hek293 cells were transfected for 36 h with pcDNA (mock) and various constructs. For the last 30 min of the incubation period cells were incubated with 0.33 μCi/ml of [4,5-3H]sphinganine. Results are the means ± S.D. of two independent experiments in which two culture dishes were analyzed for each construct.

FIGURE 8. Intracellular localization of CerS5, CerS5Δ129–140, and CerS5Δ129–135. The localization of CerS5 constructs, all HA-tagged at the C terminus, was compared with the endoplasmic reticulum marker, protein disulfide isomerase (PDI). Anti-HA is in red, and anti-protein disulfide isomerase in green. Yellow in the merged photos indicates areas of overlap of the two fluorophores. Bar, 10 μm.
(Hox)CerS, the first 15 amino acid residues of the Hox domain are missing (with the exception of proteins bearing homology to CerS2 in various organisms). The Hox domain, therefore, appears to be degenerate toward the N-terminal region of the domain, consistent with the much lower conservation in this region between species (Figs. 1 and 9).

In contrast, the C-terminal end of the Hox domain is one of the most highly conserved regions in (Hox)CerS proteins (Fig. 1), whereas the corresponding region upstream to the TLC domain in (non-Hox)CerS shows very little conservation between a wide variety of species (compare Fig. 9, A and B, and see supplemental Table 2), except for a minor preference for positively charged amino acids. Together these results imply that the conserved region at the end of the Hox domain in (Hox)CerS may be of previously unsuspected functional significance. This becomes even more likely upon comparison of this region with the Hox domain in other Hox domain-containing proteins (Fig. 10) in which this region is highly conserved. However, some critical differences exist that may impact upon the function of this domain. For instance, the invariant residue Asn-51 in other Hox domains (9) is never (with one exception) an asparagine in Hox domains in (Hox)CerS (residue 4 in Fig. 10). Asn-51 has been implicated in DNA binding, an essential feature if the Hox domain is to act as a transcription factor. Likewise, residue 58 in other Hox domains is a lysine or arginine residue in more than 90% of sequences analyzed but is replaced by proline in the Hox region of (Hox)CerS (residue 11 in Fig. 10). A number of other critical differences exist, and together these differences strongly suggest that the Hox domain in (Hox)CerS does not act as a sequence-specific transcription factor but, rather, may play an important role in, for instance, the regulation of catalytic activity.

Further support for this idea comes from the second major observation in the current study, namely that deletion or modification of this sequence leads to loss of CerS activity. The mechanism by which the two positively charged residues adjacent to the Hox domain function in CerS catalytic activity is not known, but it appears that the presence of a positively charged amino acid is required immediately before the FCES sequence (residues 141–144 in CerS5) for maximal CerS activity. All prediction programs demonstrate that Lys-134 is part of the Hox domain, and most programs suggest that Lys-140 is part of the TLC domain, with the exception of SMART, which defines Lys-140 as part of both the Hox and TLC domains. A positively charged residue (either Lys or Arg) is found immediately before FCES in all putative CerS in all organisms (Fig. 9). This renders somewhat surprising the observa-
tion that K140A retains 50% activity (Fig. 4). To resolve this, we propose that the three-dimensional structure in this region may allow another positively charged residue in the C-terminal portion of this region, such as Lys-134, to substitute for Lys-140. Remarkably, when the 12 residues of this region are used as the input for a pattern search using all residues at each position, the only proteins recognized are (Hox)CerS, with the exception of three unrelated proteins which show the lowest homology; these proteins are not recognized upon inclusion of an additional four residues at the N terminus of this region (Fig. 10 and supplemental Table 3). We, therefore, propose that this region can be used as a new motif to define (Hox)CerS and, moreover, is functionally important for CerS activity since its modification or removal leads to a loss of CerS activity.

In summary, we provide novel mechanistic insights into the mode of catalytic activity of mammalian CerS proteins and demonstrate a previously unsuspected role in CerS activity for two positively charged residues flanking the Hox and TLC domains. These residues are part of a new functional motif that can be used to define (Hox)CerS.

Acknowledgments—We thank Richard Kolesnick (Sloan Kettering Institute) for human CerS6 and Renita Hazan for help preparing plasmids.

REFERENCES

1. Futerman, A. H., and Riezman, H. (2005) Trends Cell Biol. 15, 312–318
2. Pewzner-Jung, Y., Ben-Dor, S., and Futerman, A. H. (2006) J. Biol. Chem. 281, 25001–25005
3. Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Stullards, M. C., Merrill, A. H., Jr., and Futerman, A. H. (2002) J. Biol. Chem. 277, 35642–35649
4. Riebeling, C., Allegood, J. C., Wang, E., Merrill, A. H., Jr., and Futerman, A. H. (2003) J. Biol. Chem. 278, 43452–43459
5. Mizutani, Y., Kihara, A., and Igarashi, Y. (2005) Biochem. J. 390, 263–271
6. Mizutani, Y., Kihara, A., and Igarashi, Y. (2006) Biochem. J. 398, 531–538
7. Winter, E., and Ponting, C. P. (2002) Trends Biochem. Sci. 27, 381–383
8. Venkataraman, K., and Futerman, A. (2002) FEBS Lett. 528, 3–4
9. Gehring, W. J., Affolter, M., and Burglin, T. (1994) Annu. Rev. Biochem. 63, 487–526
10. Kageyama-Yahara, N., and Riezman, H. (2006) Biochem. J. 398, 585–593
11. Spassieva, S., Seo, J. G., Jiang, J. C., Bielawski, J., Alvarez-Vasquez, F., Jazwinski, S. M., Hannun, Y. A., and Obeid, L. M. (2006) J. Biol. Chem. 281, 33931–33938
12. Guillais, I., Jiang, J. C., Vionnet, C., Roubaty, C., Uldry, D., Chuard, R., Wang, J., Jazwinski, S. M., and Conzelmann, A. (2003) J. Biol. Chem. 278, 37083–37091
13. Yu, Y., Lu, H., Pan, H., Ma, J. H., Ding, Z. J., and Li, Y. Y. (2006) Microbiol. Res. 161, 203–211
14. Hirschberg, K., Rodger, J., and Futerman, A. H. (1993) Biochem. J. 290, 751–757
15. Hulo, N., Bairoch, A., Bulliard, V., Cerutti, L., De Castro, E., Langendijk-Jeneux, P. S., Pagni, M., and Sigrist, C. J. (2006) Nucleic Acids Res. 34, 227–230
16. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
17. Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004) Genome Res. 14, 1188–1190
18. Letunic, I., Copley, R. R., Schmidt, S., Ciacchelli, F. D., Doerks, T., Schultz, J., Ponting, C. P., and Bork, P. (2004) Nucleic Acids Res. 32, 142–144
19. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
20. Lahiri, S., and Futerman, A. H. (2005) J. Biol. Chem. 280, 33735–33738
21. Vallee, B., and Riezman, H. (2005) EMBO J. 24, 730–741