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Domain homologues of dopamine β-hydroxylase and ferric reductase: roles for iron metabolism in neurodegenerative disorders?

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One of the defining characteristics of neurodegenerative diseases, including Parkinson's, Alzheimer's and Huntington's diseases, is abnormal accumulations of iron, specifically in affected areas. Following injection of iron in rat brains, a relatively selective lesion of dopamine neurons, similar to parkinsonism, occurs. These observations indicate that Fe(II)-mediated generation of free radical species, by the Fenton reaction, might contribute to the pathoetiology of these diseases. Iron is known to possess multiple roles in the biosynthesis of catecholamines in dopaminergic neurons. These include, as Fe(II), facilitating the production of dopamine from phenylalanine by tyrosine hydroxylase, and as heme, assisting the recycling of ascorbate by cytochrome b-561 required for the generation of norepinephrine from dopamine by dopamine β-hydroxylase. In this study, it is demonstrated that a human and mouse gene product, stromal cell-derived receptor 2, is a homologue of cytochrome b-561 and duodenal cytochrome b, and is thus predicted to be active as a ferric reductase. Moreover, this protein also contains a domain homologous to the N-terminal regulatory region of dopamine β-hydroxylase. These findings from sequence analysis lead to a prediction that stromal cell-derived receptor 2 is a catecholamine-regulated ferric reductase active in the brain. Dysfunction of cytochrome b-561 or stromal cell-derived receptor 2, therefore, might predispose individuals to abnormal accumulation of Fe(III) and/or generation of cytotoxic free radicals as a consequence of a rapid cycling between Fe(III) and Fe(II). The hypothesis that aberrant ferric reductase activities are involved in the progression of neurodegenerative diseases should open up new avenues of research, and possibly therapy, for these devastating diseases.

INTRODUCTION

A hallmark of neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's chorea, is a significant increase in iron in affected brain regions (1–3). Basal ganglia ferritin iron content is significantly increased in patients with AD (4). Similarly, in PD patients, iron is increased by ~35% specifically in the degenerating substantia nigra pars compacta (reviewed in 5). For PD patients this increase is due mainly to a rise in insoluble ferric [Fe(III)], rather than soluble ferrous [Fe(II)], iron (6).

Iron is a critical factor in the biosynthesis of norepinephrine and dopamine. For example, the metal is a cofactor of tyrosine hydroxylase in its conversion of phenylalanine to dopamine, a catecholamine neurotransmitter found in neurons of the central and peripheral nervous systems. Norepinephrine is converted from dopamine by dopamine β-hydroxylase, an ascorbate- and copper-dependent mono-oxygenase, primarily in the chromaffin vesicles of the adrenal gland. Within these vesicles ascorbate is recycled by importing reducing equivalents across the vesicle membrane. This is mediated by the transmembrane protein cytochrome b-561 which requires iron in the form of two molecules of heme bound to conserved histidine residues.

It has been proposed that iron and free radicals participate in neuronal cell death in the parkinsonian substantia nigra and in AD (7–9). Elevation of iron and depletion of the neuroprotective agent glutathione in the substantia nigra are correlated with loss of dopamine and stages of PD (1–3). The hydroxyl radical (OH·) and Fe(III) are generated upon the reaction of hydrogen peroxide with Fe(II). This Fenton reaction may account both for the increase in ferric iron and also for the increased production of reactive oxygen species in the substantia nigra in PD, as observed by the significant decrease in glutathione (reviewed in 5,9).

It is clear that the functions of iron, reactive oxygen species and dopamine are intimately linked in dopaminergic neurons. Furthermore, the elevation of iron in affected regions of patients with PD might implicate iron and reactive oxygen species in the etiology of this, and other neurodegenerative, disorders (7,9). Of particular relevance is a recent identification by McKie *et al.* (10) of a cytochrome b-561 homologue, termed Dcytb, as an iron-regulated ferric reductase in the mouse duodenal mucosa. Dcytb was shown to reduce insoluble ferric Fe(III) to soluble ferrous Fe(II) facilitating the uptake of ferrous iron in the small intestine. Although not suggested by McKie *et al.* (10), this observation is important in that it strongly predicts that cytochrome b-561, as a Dcytb homologue, is also a ferric reductase.

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In this study, I demonstrate a third member of the cytochrome b-561/Dcytb family of predicted ferric reductases that additionally possesses a domain homologous to the regulatory domain of dopamine β-hydroxylase. The co-occurrence of these two domains in a single polypeptide predicts a role for this protein in catecholamine-regulated reduction of ferric iron in the mammalian brain. This hypothesis has implications for understanding the relevance of elevated iron levels in neurodegenerative disorders.

RESULTS AND CONCLUSIONS

Sequence analysis of stromal cell-derived receptor 2 (SDR2)

The mouse SDR2 sequence (GeneInfo accession no. 1747306) (11) was found to contain a signal peptide (SignalP-HMM probability = 0.93) indicating that it is a secreted protein. Domain analysis using Pfam and SMART showed that the N-terminal region of the mature protein (amino acids 31–156) contains a reeler domain. The function(s) of this domain remain unknown, but its presence in two proteins involved in neuronal guidance, reeler (12) and F-spondin (13), implies a neuron-specific function.

Two further domains were detected in SDR2. The first of these represents the middle portion of SDR2 and was found to be homologous to the N-terminal regulatory domain of dopamine β-hydroxylase. This family of homologous domains will be termed dopamine β-hydroxylase homology (DoH) domains. The domain most similar in sequence to the SDR DoH domain occurs in a brain-specific protein, CG-6 (14). The *CG-6* gene was considered to be a candidate gene for Familial Dysautonomia (FD), which is characterized by the degeneration of specific neuronal populations from the peripheral nervous system. However, due to the absence of coding mutations, obvious deletions and rearrangements in four patients with FD, it was considered unlikely to be involved in this disease. This FD candidate gene is discussed below.

A PSI-BLAST search of current sequence databases with the CG-6 sequence revealed significant similarity with the regulatory domain of chicken mono-oxygenase X ($E = 3 \times 10^{-5}$; six rounds) and mouse dopamine β-hydroxylase ($E = 4 \times 10^{-8}$; seven rounds). Further analysis using hidden Markov models (HMMs) and PSI-BLAST revealed that these DoH domains occur in over 30 proteins in plants, arthropods, nematodes and vertebrates (Figs 1A and 2).

The most C-terminal domain in mouse SDR2 was found, using PSI-BLAST, to be homologous to eukaryotic cytochromes b-561. Querying the NR database with amino acids 360–592 of SDR2 demonstrated significant similarity ($E = 6 \times 10^{-4}$) with *Xenopus laevis* cytochrome b-561 by round 3. The regions of SDR2 and cytochrome b-561 that are sequence-similar contain four predicted transmembrane helices represented by stretches of consecutive hydrophobic residues. The homologous regions of SDR2 and cytochrome b-561 are unlikely to have been identified simply by chance since the multiple alignment (Fig. 1B) demonstrates the conservation, in SDR2 homologues, of two pairs of histidine residues that are thought to coordinate two heme groups in cytochrome b-561 (15).

The family of DoH domains

DoH domains were identified in four mammalian proteins (dopamine β-hydroxylase, its paralogue, mono-oxygenase X, SDR2 and CG-6). With the exception of CG-6, orthologues of each of these appear to be represented in the *Drosophila melanogaster* genome as well as four additional DoH domain-containing proteins (Fig. 2). The nematode worm, *Caenorhabditis elegans,* was found to possess a dopamine β-hydroxylase orthologue as well as a SDR2-like protein with four tandem DoH domains (Fig. 2). The plant, *Arabidopsis thaliana*, possesses 11 versions of proteins containing a DoH domain positioned N-terminal to a cytochrome b-561-like domain, as well as a single DoH domain protein [Air12, whose mRNA accumulates during auxin-induced lateral root formation (16)].

The identification of the DoH domain family does not readily allow prediction of this domain's functions. However, two observations are consistent with a catecholamine-binding function for DoH domains. First, the phyletic distribution of DoH domains in plants, flies, nematode worm and mammals mirrors the distribution of organisms that synthesize dopamine and other catecholamines. Secondly, it is known that overexpression of dopamine β-hydroxylase in mice results in unexpected steady-state levels of norepinephrine (17). One mechanism that might account for this observation is strong negative feedback mechanism with norepinephrine binding to the dopamine β-hydroxylase DoH domain thereby allosterically inhibiting the protein's enzymatic activity. This is consistent with the close spatial proximity of the regulatory and catalytic domains in the enzyme as shown by their covalent attachment by a disulphide-bridge (18). This regulatory mechanism would be in addition to the known inhibition of tyrosine hydroxylase activity by dopamine (19).

As discussed above, *CG-6* was previously discounted as a candidate gene for FD (14). Subsequently, two groups detected in FD patients a splicing mutation in *IKBKAP* (20,21), a *CG-6* neighbouring gene on chromosome 9q31. However, the presence of a DoH domain in CG-6 might suggest an additional contributing gene to this disorder. If mutations in *IKBKAP* were the sole basis to FD it is difficult to account for the known decrease in the synthesis of norepinephrine in FD patients (22), thought to be due to decreased levels of dopamine β-hydroxylase (23). On the other hand if FD patients were to possess additional mutations in *CG-6* this might account for these observations on the basis that dopamine β-hydroxylase and CG-6 both possess DoH domains that are predicted to regulate the biosynthesis of epinephrine and norepinephrine.

The family of cytochrome b-561 domains

Six cytochrome b-561 homologues were identified in human or mouse sequences. These were: cytochrome b-561, Dcytb (10), SDR2, 101F6 [a putative tumour suppressor, see GenBank sequence GeneInfo (GI) 5901884], a 101F6 paralogue (GI 12837950) and a sixth gene on human chromosome 11q12.3. The latter gene is represented in part by the human expressed sequence tag (EST) BG685076. Similar numbers of cytochrome b-561 homologues were detected in *C.elegans* (6) and *Drosophila* (8) and *Arabidopsis* (15) (Fig. 2).

Each of these proteins is likely to possess ferric reductase activity, by virtue of their homology to Dcytb and their

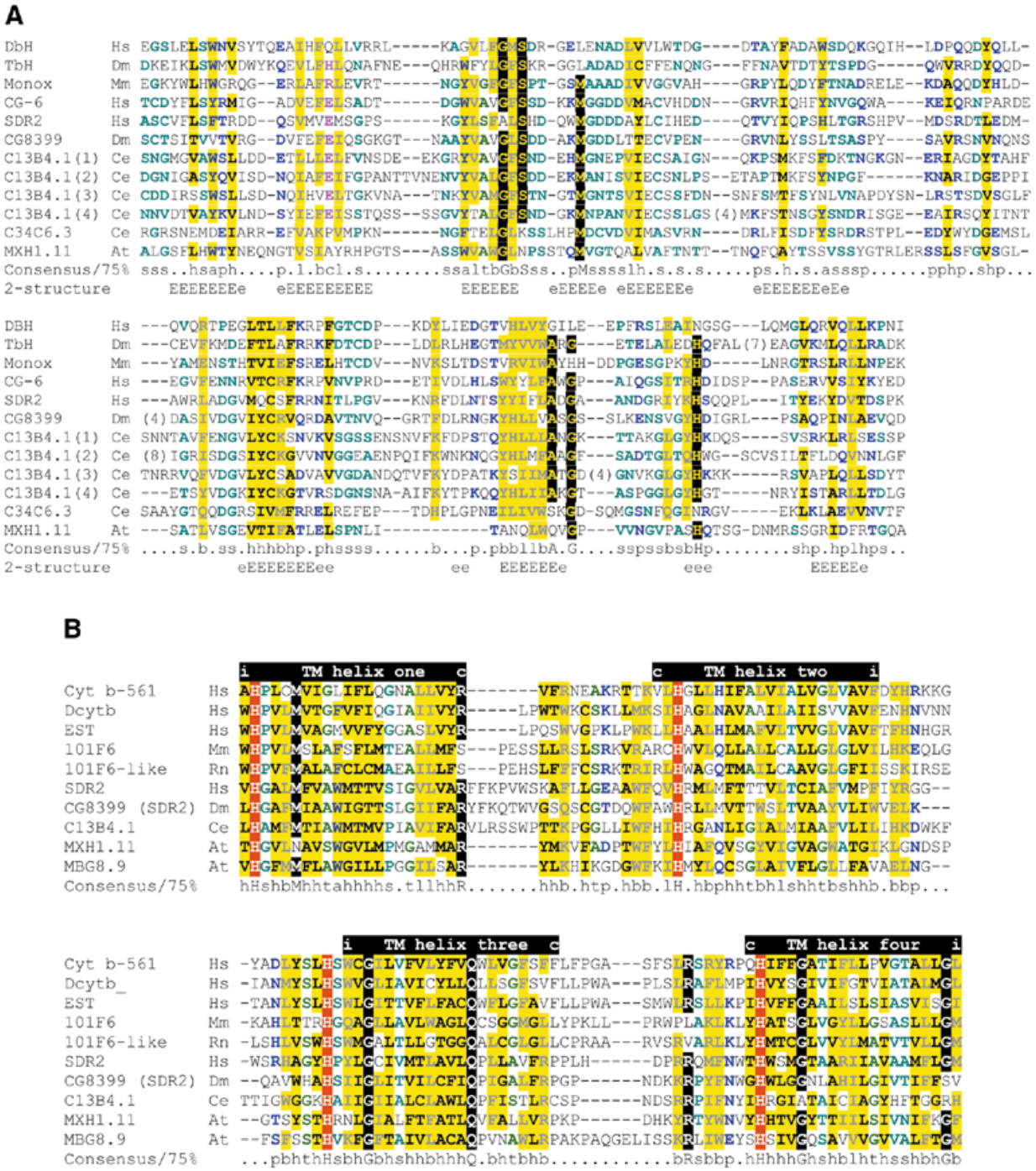

Figure 1. Multiple sequence alignments of representative (**A**) DoH domains, and (**B**) cytochrome b-561 domains, coloured according to a 75% consensus using CHROMA (L.Goodstadt and C.P.Ponting, unpublished data). Species abbreviations: At, *A.thaliana* (thale cress); Ce, *C.elegans* (nematode worm); Dm, *D.melanogaster* (fruit fly); Hs, *Homo sapiens*; Mm, *Mus musculus* (mouse); Rn, *Rattus norvegicus* (rat). Consensus abbreviations (amino acids): a, aromatic (FHWY); b, big (EFHIKLMQRWY); c, charged (DEHKR); h, hydrophobic (ACFGHILMTVWY); l, aliphatic (ILV); p, polar (CDEHKNQRST); s, small (ACDGNPSTV); t, tiny (AGS). Multiple alignments are available from SMART. (**A**) Secondary structures predicted at expected accuracies of >82% (E) or >72% (e) are indicated below the alignment (E/e, extended or β-strand structure). Protein abbreviations, GI numbers and residue numbers (if appropriate): Dbh, dopamine β-hydroxylase (GI 118791; 42–188); TbH, tyramine β-hydroxylase (GI 1296519; 95–246); Monox, mono-oxygenase X (GI 9988950; 34–176); CG-6, candidate gene-6 (GI 6760015; 169–312); SDR2, stromal cell-derived receptor 2 (GI 9797668); CG8399 (GI 7303004; 241–391); C13B4.1 (GI 7495914; 38–192, 217–377, 398–556, 586–739); C34C6.3 (GI 7496990; 305–453); MXH1.11 (GI 9758647; 48–194). (**B**) Predicted transmembrane helices are indicated for cytochrome b-561 homologous domains with assigned intravesicular (i) and cytoplasmic (c) ends. Conserved histidine residues that are proposed to bind heme are highlighted as white-on-red, whereas other amino acids conserved in at least 75% of sequences are shown as white-on-black. Protein abbreviations, GI numbers and residue numbers (if appropriate): Cyt b-561, cytochrome b-561 (GI 1345640; 52–179); Dcytb, duodenal cytochrome b (GI 10440158; 49–175); EST, expressed sequence tag (GI 13916473); 101F6 (GI 4928227; 47–178); 101F6-like (GI 7171993); SDR2, stromal cell-derived receptor 2 (GI 9797668); CG8399 (GI 7303004; 402–529); C13B4.1 (GI 7495914; 753–885); MXH1.11 (GI 9758647; 210–333); MBG8.9 (GI 9758263; 688–815).

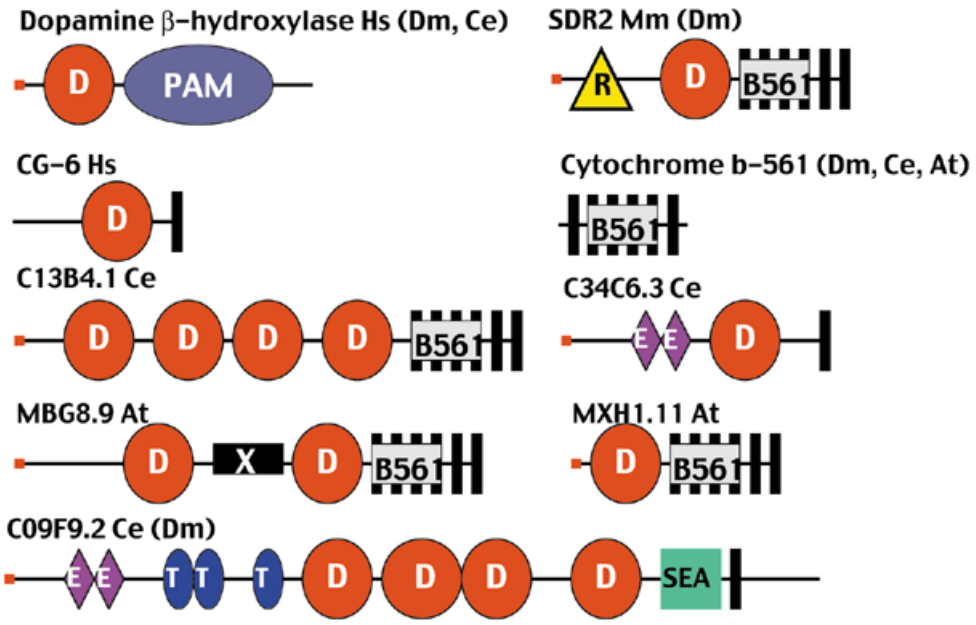

Figure 2. Schematic representation of the domain architectures of representative DoH and cytochrome b-561 homologous domains from *H.sapiens* (Hs), *M.musculus* (Mm), *D.melanogaster* (Dm), *C.elegans* (Ce) and *A.thaliana* (At). Proteins are drawn approximately to scale. Abbreviations of species names in parentheses indicate the existence of proteins in these species with identical or closely similar domain architectures. Abbreviations: B561, cytochrome b-561 domain; D, DoH domain; E, epidermal growth factor-like domain; PAM, peptidyl-glycine α-amidating mono-oxygenase (copper type II, ascorbate-dependent mono-oxygenase) homology; R, reelin domain; SEA, sea urchin sperm protein, enterokinase, agrin domain; T, trypsin inhibitor-like (cysteine-rich) domain; X, a previously unidentified domain present also in *Drosophila* CG6217, CG14682 and CG12492. Red lines at the N-terminus of proteins represent signal peptide sequences.

absolute conservation of four heme-binding histidine residues (Fig. 1B). A pair of these conserved histidine residues are predicted to occur on the intravesicular side of the cytochrome, with the second pair on the cytoplasmic side (Fig. 1B); these represent the likely two heme-binding centres of the cytochrome (15). In addition, the strong conservation of two arginine residues, one methionine residue and one glutamine residue strongly suggests their involvement in heme-binding and/or catalysis (Fig. 1B).

Of the six cytochrome b-561 homologues in mammals, three lines of evidence suggest that SDR2, as well as cytochrome b-561 itself, might be involved in ascorbate regeneration and Fe(III) reduction, coupled to dopamine β-hydroxylase activity. First, SDR2 ESTs have been characterized from mouse brain and hypothalamus cDNA libraries (ESTs AU067508 and AW494339). Secondly, as discussed previously, putative catecholamine-binding DoH domains are found in both SDR2 and dopamine β-hydroxylase. Finally, Kamensky and Palmer (24) recently reported that chromaffin granule membranes contain at least three heme centres, rather than the two centres present in cytochrome b-561. The additional heme centre(s) might be accounted for by the presence of a cytochrome b-561 homologue, namely SDR2.

Possible roles for cytochrome b-561 and SDR2 in the progression of neurodegenerative disorders

Iron is believed to be an initiation factor in the parkinsonian degeneration of neurons in the substantia nigra. A causal link between high levels of iron and neurodegeneration is demonstrated by the relatively selective lesion of dopamine neurons, similar to parkinsonism, in rats following injection of iron; addition of the iron chelator desferrioxamine protects against this toxic effect (25,26).

Further experiments have demonstrated a complex interplay between Fe(III), Fe(II), dopamine and reactive oxygen species in the development of PD (7). One model is that free radical generation contributes significantly to neuronal cell death in the parkinsonian substantia nigra (7–9). The key ingredient to free radical generation in this model is Fe(II). This might be produced either within a Fe(III)-dopamine complex, or for Fe(III) bound to ferritin upon the action of 6-hydroxydopamine, or by ascorbate. On the other hand, the sequence-based findings described here suggest that generation of Fe(II) might occur directly by a ferric reductase such as cytochrome b-561 or SDR2.

These findings might also explain the correlation between the increase in Fe(III) and the decrease in dopamine observed in PD patients since decreased ferric reductase activity of cytochrome b-561 and/or SDR2 might lead to a slow accumulation of insoluble Fe(III), rather than soluble Fe(II), in the substantia nigra. In addition, if the ferric reductase activity of SDR2 (but not cytochrome b-561) were to be inhibited by catecholamines binding to its DoH domain, then a reduction in dopamine levels would result in rapid cycling between Fe(III) and Fe(II) with concomitant generation of free radicals via the Fenton reaction.

An increase in the Fe(II) concentration might also explain decreased levels of dopamine in affected tissue. Tyrosine hydroxylase requires Fe(II) as cofactor for activity and is inactive upon the addition of Fe(III) (27). Thus, increases in

Fe(III) at the expense of Fe(II) are predicted to result in reduced dopamine biosynthesis.

The identification of a domain family whose proposed function is the binding of catecholamines might be important for the understanding of diseases other than PD. Abnormal metabolism of dopamine and norepinephrine is involved in other neuronal disorders such as AD, schizophrenia, Tourette's syndrome, Huntington's chorea, ischaemia and an orthostatic syndrome caused by dopamine β-hydroxylase deficiency (28). Furthermore, the extension of the proposed ferric reductase cytochrome b-561 family to six members in mammals might assist future insights into disorders of iron metabolism. Finally, as increased brain iron is also a symptom of Huntington's disease, dentatorubral pallidoluysian atrophy and AD, it is possible that in-depth investigations into the mutations and functions of DoH and cytochrome b-561 families might further illuminate the molecular bases of these devastating diseases.

MATERIALS AND METHODS

Protein sequence database searches used the position-specific iterative BLAST (PSI-BLAST; version 2) method (29). Briefly, an amino acid sequence is compared against a non-redundant protein sequence database (NR) and the pairwise alignments with highest scores (*S*) are provided with estimates of *E*, the number of different alignments with scores equivalent to or better than *S* which are expected to occur in the database search purely by chance. Those sequences that are aligned with *E*-values less than the default threshold ($E < 0.002$) are multiply aligned with the query sequence and a profile, or position-specific scoring matrix, is calculated. This is a numerical representation of the multiple sequence alignment and is used as the query for a subsequent search of the database. Additional iterations, that accumulate predicted homologues with $E < 0.002$ and calculate a profile that is compared with a database, are performed until no new homologues ($E < 0.002$) are detected. These searches employed the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/psiblast.cgi) and composition-based statistics (http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html#composition_based_statistics). A consequence of the use of these statistics is that it is unlikely that compositionally biased transmembrane regions would lead to inaccurate prediction of homologues.

Initially, multiple alignments of domain homologues were constructed using Clustal-W (30) and manually edited using Seaview (31). The alignment of more divergent members of a family was guided by comparison of an HMM (32) with sequences that were found to be homologues according to PSI-BLAST searches. Secondary structure predictions from multiple alignments were provided by PHD (33).

Predictions of signal peptides used the SignalP-HMM method (34) at http://www.cbs.dtu.dk/services/SignalP-2.0/. Domain identification used the Pfam (http://www.sanger.ac.uk/Pfam/) (35) and SMART (http://smart.embl-heidelberg.de/) (36) web-based resources. Human genome and expressed sequence tag sequences were queried using BLAST, Ensembl (http://www.ensembl.org/) and dbEST (http://www.ncbi.nlm.nih.gov/dbEST/).

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