The CC2D1A, a member of a new gene family with C2 domains, is involved in autosomal recessive non-syndromic mental retardation


Background: The molecular basis of autosomal recessive non-syndromic mental retardation (NSMR) is poorly understood, mostly owing to heterogeneity and absence of clinical criteria for grouping families for linkage analysis. Only two autosomal genes, the PRSS12 gene on chromosome 4q26 and the CRBN gene on chromosome 3p26, have been shown to cause autosomal recessive NSMR, each gene in only one family.

Objective: To identify the gene causing autosomal recessive NSMR on chromosome 19p13.12.

Methods: The candidate region established by homozygosity mapping was narrowed down from 2.4 Mb to 0.9 Mb on chromosome 19p13.12. A protein truncating mutation was identified in the gene CC2D1A in nine consanguineous families with severe autosomal recessive NSMR. The absence of the wild type protein in the lymphoblastoid cells of the patients was confirmed. CC2D1A is a member of a previously uncharacterised gene family that carries two conserved motifs, a C2 domain and a DM14 domain. The C2 domain is found in proteins which function in calcium dependent phospholipid binding; the DM14 domain is unique to the CC2D1A protein family and its role is unknown. CC2D1A is a putative signal transducer participating in positive regulation of the kinase/NFκB cascade. Expression of CC2D1A mRNA was shown in the embryonic ventricular zone and developing cortical plate in staged mouse embryos, persisting into adulthood, with highest expression in the cerebral cortex and hippocampus.

Conclusions: A previously unknown signal transduction pathway is important in human cognitive development.

METHODS

Patients

The study was reviewed and approved by the human subjects committee of the Rabin Medical Centre. The initial clinical presentation in all affected family members was psychomotor developmental delay during early childhood. All affected individuals had no or only single words and were severely mentally retarded, whereas their general physical examination was normal. There were no autistic features or seizures. The general appearance was normal, with no dysmorphic features; height, weight, and head circumference were normal in all except one subject who had progressive microcephaly. Neurological examination of the patients was normal.

Formal psychological evaluation was carried out on one patient at the age of eight years. He was assessed by the Leiter International Performance Scale and was found to function in the severely mentally retarded range.

Microsatellite marker analysis

DNA was isolated from the blood samples by standard methods. Four hundred microsatellite markers, spaced at 10 cM intervals, from the ABI PRISM linkage mapping set, version 2.5 (Applied Biosystems, Foster City, California, USA) were typed.

Abbreviations: NSMR, non-syndromic mental retardation
were amplified by multiplex polymerase chain reaction (PCR), using standard protocols. Amplified markers were electrophoresed on an ABI 3700 DNA capillary sequencer and were analysed with Genescan and Genotyper software (Applied Biosystems).

**Sequencing of candidate genes**

We identified the genes in the candidate locus by means of available databases (NCBI and UCSC genome browser). Sequencing of the candidate genes was undertaken with primer sets designed using standard software Primer 3. All exons, including exon–intron junctions, were amplified from genomic DNA with primers designed from the genomic sequences available from NCBI and UCSC genome browser.

Both strands of the PCR products were sequenced with BigDye Terminators (Applied Biosystems) on an ABI 3100 sequencer. Sequence chromatograms were analysed using SeqScape software, version 1.1 (Applied Biosystems). We initially tested one affected individual, one heterozygous parent, and a non-carrier sibling (as defined by haplotype analysis).

**Molecular analysis of the CC2D1A gene**

On the basis of the predicted genomic sequence, the 21 primer pairs were used for PCR amplification of exons and splicing junctions (primer sequences available on request).

**CC2D1A** deletion mutation screening was undertaken on genomic DNA by PCR using the following primers:

---

**Figure 1** Fine mapping of the candidate region on 19p13.12. The genotypes of the pedigrees with autosomal recessive NSMR. Black symbols in the pedigree represent affected individuals. The boxed regions indicate the homozygous regions in each affected individual. The polymorphic markers are shown on the left. Arrows indicate informative recombinations.
for normal alleles: primer A (forward): GTGGATGTGTCGCTGAATTGC; primer B (reverse): CAAGCGATCCTCCCATCTT; for mutant alleles: primer A (forward): GTGGATGTGTCGCTGAATTGC; primer C (reverse): TACAGTCCTCCGCAAACCTTT. Primer A was derived from exon 13 (upstream of the deletion), primer B from intron 14 (within the deletion), and primer C from exon 19 (downstream of the deletion).

**RNA isolation**
We obtained RNA from the blood of the affected individual by use of Trizol reagent and carried out reverse transcriptase reactions with both random hexamers and oligo dT primers in order to obtain cDNA.

**Western blot**
Whole cell extracts were separated on 7.5% SDS-PAGE and analysed by immunoblotting with CC2D1A specific antibody. The cell proteins were prepared. Briefly, cells were washed twice with phosphate buffered saline (PBS) and lysed at 4°C with lysis buffer (0.6% NP40 and protease inhibitors). After 15 minutes of incubation, cell lysates were centrifuged for five minutes at 16 000 x g and supernatants were collected. Protein concentrations were determined using the BCA protein assay reagent kit (Pierce Biotechnology, Rockford, Illinois, USA). Whole cell proteins (approximately 20 µg) were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween20) for 30 minutes at room temperature and subsequently incubated with anti-CC2D1A rabbit polyclonal antibody (Bethyl Laboratories, Montgomery, Texas, USA) diluted 1:20 000, or rabbit polyclonal anti-emerin diluted 1:5000, or mouse monoclonal antibodies anti-NFκB p50 diluted 1:1000/p65 diluted 1:5000 (Santa Cruz Biotechnology, Santa Cruz, California, USA). After washing and subsequent incubation for one hour with horseradish peroxidase coupled to anti-rabbit IgG or mouse IgG, respectively (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), immunoreactive proteins were visualised by an ECL (enhanced chemiluminescence super signal) detection kit (Pierce Biotechnology). Antibody to emerin protein (Santa Cruz Biotechnology) was used to demonstrate the loading level.
Immunofluorescence
Human bone osteosarcoma line U2OS cells were grown on cover slips overnight. The cells were fixed with ice cold methanol (five minutes) and ice cold acetone (five minutes), washed with tris buffered saline (TBS; 100 mM Tris-HCl pH 7.5, 150 mM NaCl) and blocked using 5% skimmed milk in TBS containing 0.1% Tween 20 (TBS-T) for 30 minutes. Incubations with primary (rabbit polyclonal anti-CC2D1A (Bethyl Laboratories; 1:100 dilution), and secondary (Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories; 1:500 dilution) antibodies were carried out in blocking solution for one hour each. Between and after the incubation with the antibodies, cells were washed three times with TBS-Tween. The cover slips were mounted using Fluoromount-G (SouthernBiotech, Birmingham, Alabama, USA) and the cells photographed after fluorescent microscopy analysis.

Immunoprecipitation
A sample of approximately 200 µg total protein from the lymphoblasts of a patient and a control subject were incubated with rabbit anti-CC2D1A antibody (diluted 1:100) overnight, with rotation at 4°C. Subsequently 50 µl anti-rabbit IgG (whole molecule) agarose antibody (Sigma Chemical Company, St Louis, Missouri, USA) was added and the mixture was incubated for one hour at room temperature. The agarose beads were washed once with a lysis buffer and three additional times with PBS. Absorbed proteins were solubilised with SDS-PAGE sample buffer and subjected to electrophoresis on 12% polyacrylamide gels and immunobotted as described in the western blot method.

In situ hybridisation
Non-radioactive in situ hybridisation was carried out as previously described in frozen mouse brains, using digoxigenin (DIG)-labelled cRNA probes. Briefly, the murine CC2D1A probes were generated by PCR from IMAGE clone 5363547 (BC027028). Probes were generated at the following nucleotide (nt) sequence positions in the CC2D1A cDNA: probes CC2D1A-1 (nt 38–506; 469 bp) and CC2D1A-2 (nt 2657–3021; 365 bp; 3'-UTR). All CC2D1A nucleotide probe sequences were from nucleotides downstream of the ATG start site. The probes were made from PCR amplified sequences using primers constructed to amplify the sequences described above (primer sequences are available upon request). All forward primers contained T3 promoter sequences using primers constructed to amplify the start site. The probes were made from PCR amplified sequences from nucleotides downstream of the ATG translation of the prediction. Tetraodon2 is a result of translation of the prediction. The phylogenetic analysis included multiple alignment using ClustalX and dendorgram creation with MEGA2 using neighbour joining. Accession numbers for the family members are: XP542027 (Dog1), XP237884 (Rat1), NP666082 (Mouse1), XP_614352 (Cow1), CAG06037 (Tetraodon1), XP_513407 (Chimpanzee2), XP588670 (Cow2), XP233342 (Rat2), NP796019 (Mouse2), CAG077113 (Tetraodon2), and MGC68887 (Xenopus laevis). The protein Chimpanzee1 was defined from the chimpanzee genome by aligning the human mRNA using Sim4 and translation of the prediction. Tetraodon2 is a result of Genescan prediction the region chr15_random: 1575804-1615381, which contains 14 genes and T7 promoter sequences on their 5’ ends in order to drive the transcription of the cRNA antisense probes, and all reverse primers contained T7 promoter sequences on their 5’ ends in order to drive the transcription of the cRNA antisense probes.

RESULTS
Refinement of the candidate region
A common homozygous disease bearing haplotype for the polymorphic markers RFX1 and D19S840 defined a critical region of 0.9 Mb between the markers D19S564 and D19S547 in the four original and five additional families, with a total of 16 affected and 48 unaffected individuals, all living in the same village and bearing the same family name (fig 1). The presence of the common disease haplotype suggests that the disease is caused by a single mutation derived from a single ancestral founder in all the families. A minimal candidate region contains 14 genes (fig 2A).

Mutation analysis
All the genes in the candidate region were analysed. Sequencing of exons 14, 15 and 16 of the CC2D1A gene
showed failure of amplification in all the affected family members as opposed to successful amplification in heterozygous and homozygous normal individuals. Sequencing of the cDNA with the primers derived from exons 13 and 17 confirmed complete deletion of exons 14, 15, and 16; sequencing of the genomic DNA with the primers flanking the exon–intron junctions for exons 13 and 17 revealed the deletion of 3589 nucleotides beginning in intron 13 and ending in intron 16 (IVS13 IVS16del). The deletion causes removal of aa 408 to 547 and introduces a frameshift of the encoded protein immediately after the deletion, creating a nonsense peptide of 30 aa and a stop codon at position 438 of the mutant protein (G408fsX437). In each pedigree analysed, the CC2D1A mutation segregated with the disease; affected patients displayed homozygous CC2D1A mutations, whereas parents who were clinically normal displayed heterozygosity for a normal and a disease allele, consistent with recessive inheritance (fig 2B and 2C). The mutation abolishes DM14 and C2 domains of the gene (fig 2D). The mutation was not observed in 300 control chromosomes including 100 Jewish and 50 Israeli Arab individuals.

**Figure 4** In situ hybridisation of the CC2D1A mRNA. Expression of CC2D1A mRNA is seen in murine brain from E12 into adulthood. CC2D1A mRNA expression is found throughout the ventricular zone progenitor cells and developing cortical plate. Later in development at E16, CC2D1A mRNA is found throughout the cortical plate and developing hippocampus. During the postnatal period of brain development (P3), CC2D1A mRNA expression is widely expressed throughout the brain, but particularly in the cerebral cortex and hippocampus, especially CA3 of the hippocampus. In adult brain, the expression of CC2D1A mRNA is widespread but appears restricted mostly to neurones. The scale bar in the E12, E16, and P3 panels is 500 microns, and the one in the adult panel 1 mm. CBL, cerebellum; CC, corpus callosum; CP, cortical plate; CTX, cerebral cortex; GE, ganglionic eminences; HPC, hippocampus; OB, olfactory bulb; STR, striatum; TH, thalamus.
Figure 5  CC2D1A protein family. (A) A phylogenetic analysis of the CC2D1A protein family. The two invertebrate branches, A and B, are emphasised by the grey boxes. (B) A multiple alignment of the C2 domain (upper panel) and one of the DM14 domains (lower panel). Strongly conserved positions are marked on top, where '*' indicates positions that are occupied by a single amino acid, while ':' and '.' are for positions where a conserved substitution have occurred. The conservation graph is shown below. Note that some positions in the aczonin protein were truncated to allow a better alignment of the C2 domain. These are marked by the '-' sign.
**Protein analysis**

We confirmed the absence of the wild type protein product by testing patient derived lymphoblastoid cells by western blot analysis with CC2D1A antibody, which detects the N-terminus of the protein, and found that antibodies identified a 104 kDa band in normal individuals but not in the patients; the smaller protein, most probably representing the truncated protein, was observed in the patients (fig 3A).

To determine the subcellular localisation of CC2A1 protein, immunolabelling experiments were carried out by using polyclonal antibody U205S cells (fig 3B). CC2D1A protein is distributed throughout the cytoplasm; no signal was detected in the nuclei. It is a soluble protein, as no transmembrane regions are detected. The absence of signal peptide in the protein sequence suggests that it is not secreted.

We examined possible direct interactions between CC2D1A protein and the p65 and p50 subunits of the NFκB complex by using co-immunoprecipitation of the endogenous proteins in control lymphoblastoid cells. CC2DA1 protein does not interact directly with the NFκB p65 subunit or the p50 subunit in vivo (data not shown), as detected by immunoprecipitation.

**Expression analysis**

We carried out in situ hybridisation in staged mouse embryos to determine the spatial expression patterns of CC2D1A during brain development (fig 4). CC2D1A mRNA was observed at E12 throughout the ventricular zone and developing cortical plate and ganglionic eminences. By E16, CC2D1A mRNA was found throughout the mouse brain, but most strongly in the cortical plate. At postnatal (P) day 3, CC2D1A mRNA continued to be widely expressed with strong expression in the cerebral cortex and hippocampus (particularly in the hippocampal CA3 region). Continued expression was observed in the brain into adulthood.

**Gene structure and phylogenetic analysis**

The CC2D1A gene (GenBank accession number NM_017721) covers 37 kb of genomic DNA on 19p13.12. It encodes 3715 bp long mRNA, containing 31 exons. At least 12 splice variants have been identified (UCSC genome browser). The coding sequence is 2893 bp long. The gene is widely expressed in various tissues. The CC2D1A protein contains 950 amino acids (National Center for Biotechnology Information (NCBI) Entrez Protein accession number NP_060191), although a shorter isoform of the protein exists, which contains only 388 amino acids. A search against the National Centre for Biotechnology Information (NCBI) Entrez Protein accession number NP_060191), although a shorter isoform of the protein exists, which contains only 388 amino acids. A search against the NCBI protein database showed that the protein family carries two conserved motifs. The most conserved motif is a C2 domain (IPR0008973, IPR006608, IPR000008). The other motif, DM14, is unique to the CC2D1A protein family and its role is unknown.

A phylogenetic analysis of the CC2D1A protein family is shown in fig 5A. All protein family members are predicted and their function is unknown. The two invertebrate branches were inferred, using the mouse protein aczonin (presynaptic cytomatrix protein) as an out group. Sequence similarity searches of the CC2D1A protein, using BLASTP against swiss-prot and non-redundant databases, revealed similarity to many potential orthologues in mammals, in other vertebrates (Xenopus laevis, tetraodon), as well as in invertebrates (Drosophila melanogaster and Caenorhabditis elegans). No candidate orthologue was found in the yeast genome. In vertebrates the gene was duplicated, as two paralogs are found in the tetraodon genome and the mammalian genome. Although only one copy was found in the frog genome, this is probably because the full genome sequence was yet not determined. A multiple alignment of the C2 domain and one of the DM14 domains is shown in fig 5B.

**DISCUSSION**

In this study we have identified a protein truncating mutation in the novel CC2D1A gene with unknown function causing autosomal recessive NSMR. The protein has two main isoforms containing 950 amino acids and 388 amino acids. The shorter isoform of the protein does not contain exons 14 to 16, which are deleted in the patients. Thus only the long protein isoform seems to play a central role in disease pathogenesis in the patients. C2 motif, which is located at the C-terminus of CC2D1A at positions 661–762, is found in proteins which function in calcium dependent phospholipid binding, where the C2 domain itself participates in the binding pocket of the Ca2+ cation. Proteins which have a C2 domain include phospholipases, protein kinases C, and also the synaptotagmin protein family. Synaptotagmins are synaptic vesicle membrane trafficking proteins. The role of the other motif, DM14, is unknown. It repeats four times in the human CC2D1A sequence but only three times in the Caenorhabditis elegans orthologue sequence. The DM14 motif also appears only in the long isoform of CC2D1A, while the C2 domain, which is present in both isoforms, probably plays a central role in its protein activity. The protein truncating mutation in our patients abolishes one of the four DM14 domains and the C2 domain. The absence of signal peptide in the protein sequence suggests that it is not secreted. Phylogenetic analysis shows similarity to many potential orthologues in mammals, in other vertebrates, and in invertebrates, but no candidate orthologue was found in the yeast genome.

Matsuda et al originally identified CC2D1A as a putative signal transducer participating in positive regulation of IκB kinase/NFκB cascade. Their large scale study was designed to identify human genes that activate the NFκB and MAPK signalling pathways, and they reported that the CC2D1A gene was found to induce by at least fourfold the transcription of a luciferase reporter gene driven by a promoter containing the consensus NFκB binding sites. CC2D1A protein does not directly interact with the NFκB p65 subunit or the p50 subunit in vivo as detected by immunoprecipitation, suggesting that NFκB expression is regulated by the CC2D1A gene through other signalling proteins that belong to the IKK/IκB dependent or the IKK/IκB independent pathway. Functional NFκB complexes are present in all cell types in the nervous system, including neurons, microglia, oligodendrocytes and astrocytes. Stimulation of glutamate receptors and membrane depolarisation stimulate NFκB activation in neurones, probably through a calcium mediated mechanism. NFκB is also activated in association with long term potentiation of synaptic transmission, a process believed to be related to learning and memory. Electrophysiological measurements of synaptic plasticity in hippocampal cells from mice lacking tumour necrosis factor alpha receptors suggest an important function of NFκB in the long term depression (LTD) of synaptic transmission. Along with the potential role of NFκB in neuronal function in the brain, there is an indication that NFκB might be important for neuronal development based on the pattern of NFκB activity in the brain during mouse development. Basal synaptic input in mature mouse hippocampal neurones activates the NFκB transcription factor and induces κB DNA binding activity, consisting of p50:p65 and p50:p50 dimers. Mice lacking p65 show a selective learning deficit in the spatial version of the radial arm maze. These observations suggest that long term changes to adult neuronal function caused by synaptic stimulation can be regulated by NFκB nuclear translocation and gene activation.
The expression patterns of the CC2D1A mRNA appear to be restricted mostly to the ventricular zone progenitors and neurons. Given these expression patterns in the cerebral cortex and hippocampus and the phenotype in patients, CC2D1A appears to be important in cognitive development in humans.

**ELECTRONIC DATABASE INFORMATION**

Accession numbers and URLs for data presented in this paper are as follows:

- UCSC Genome Browser, http://genome.ucsc.edu/ (May 2004 build)

**ACKNOWLEDGEMENTS**

We are grateful to the families who participated in this study and clinical nurses Hitam and Inshihar. We thank Dr D Gurevitch for establishing the lymphoblastoid cell line. We also thank Dr G Halpern for her help with editing the manuscript and Rafi Rainshtein for his help with preparing the figures. This study was funded by the Yeshaya Horovitz grant for Neurogenetics research and Israel Science Foundation (grant No 690/04).

Authors’ affiliations

L Basel-Vanagaite*, E Taub, M Shoihat, Department of Medical Genetics, Rabin Medical Centre, Beilinson Campus, Petah-Tikva, Israel
R Attia*, M Yahav, L Anteki, N Magal, V Drasinoever, A Alkelai, Felsenstein Medical Research Centre, Petah Tikva, Israel
R J Ferland, C A Walsh, Howard Hughes Medical Institute, Bethesda Israel Deaconess Medical Center, and Department of Neurology, Harvard Medical School, Boston, Massachusetts, USA
T Olender, Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel
R Straussberg, Neurogenetic Clinic, Schneider Children’s Medical Centre of Israel, Petah Tikva, Israel
D Bercovich, Human Molecular Genetics and Pharmacogenetics, Migal - Galilee Bio-Technology Centre, Kryiat-Shmona, and Tel Hai Academic College, Israel
G Rechavi, A J Simon, Sheba Cancer Research Centre, Institute of Haematology, The Chaim Sheba Medical Centre, Tel Hashomer, Israel

*These two authors contributed equally to this work.

Competing interests: none declared.

**REFERENCES**


