Expression and imprinting of **MAGEL2** suggest a role in Prader–Willi syndrome and the homologous murine imprinting phenotype

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Prader–Willi syndrome (PWS) is caused by the loss of expression of imprinted genes in chromosome 15q11–q13. Affected individuals exhibit neonatal hypotonia, developmental delay and childhood-onset obesity. Necdin, a protein implicated in the terminal differentiation of neurons, is the only PWS candidate gene to reduce viability when disrupted in a mouse model. In this study, we have characterized MAGEL2 (also known as NDNL1), a gene with 51% amino acid sequence similarity to necdin and located 41 kb distal to NDN in the PWS deletion region. MAGEL2 is expressed predominantly in brain, the primary tissue affected in PWS and in several fetal tissues as shown by northern blot analysis. MAGEL2 is imprinted with monoallelic expression in control brain, and paternal-only expression in the central nervous system as demonstrated by its lack of expression in brain from a PWS-affected individual. The orthologous mouse gene (Magel2) is located within 150 kb of Ndn, with imprinted and paternally expressed, yielding 4.5 kb transcripts. We hypothesize that, although loss of necdin expression may be critical to abnormalities in brain development and dysmorphic features in individuals with PWS, loss of MAGEL2 may be critical to abnormalities in brain development and dysmorphic features in individuals with PWS.

**INTRODUCTION**

Prader–Willi syndrome (PWS) is a neurobehavioral disorder characterized by severe hypotonia and failure to thrive in infancy followed by hyperphagia and developmental delay (1). Approximately 70% of affected individuals have ~4 Mb cytogenetic deletion of their paternal 15q11–q13 region, while the remainder have maternal uniparental disomy, submicroscopic deletions or other rearrangements. PWS is caused by the absence of expression of one or more paternally expressed genes that are regulated by an imprinting center (2,3). A recently created transcription map containing the paternally expressed genes SNRPN (4), IPW (5), ZNF127 (6) and NDN (7,8) and 22 expressed sequence tags (ESTs) suggests that at least 13 paternally expressed transcripts are located within the PWS/AS deletion region (9). However, point mutations in a single gene have not been causally associated with PWS, suggesting that PWS is a contiguous gene syndrome.

We now describe our characterization of a necdin-like gene (NDNL1), also independently identified and named MAGEL2 (10). MAGEL2 is located 41 kb telomeric to NDN, within the PWS deletion region. The mouse ortholog, Magel2, is located in the region of conserved synteny on mouse chromosome 7 and within 150 kb of Ndn. The human and mouse necdin-like gene proteins are 529 and 525 amino acids, respectively, and share ~50% similarity with necdin protein. In both species, the necdin-like gene is primarily expressed in developing brain, imprinted and paternally expressed, yielding 4.5 kb transcripts. We found that, like NDN, MAGEL2 is expressed from only one allele in control brain, but is not expressed in PWS-affected brain. Finally, we examined the expression of Magel2 and Ndn in the developing mouse brain, and postulate that it may play a role in PWS and the homologous mouse imprinting phenotype.

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RESULTS

Characterization of MAGEL2 and Magel2

We identified MAGEL2 by scanning the genomic sequence for the P1-derived artificial chromosome (PAC) clone pDJ181P7 (GenBank accession no. AC006596) containing NDN. We used content mapping of NDN, MAGEL2 and D15S11 in the yeast artificial chromosomes (YACs) 925C12 and 959G3 (7) to show that MAGEL2 is telomeric to NDN (Fig. 1), experimentally confirming a previous hypothesis (10). MAGEL2 contains a series of head-to-tail tandem repeats, consisting of 27 tandem copies of a 30 bp repeat 5′ to a predicted CpG island, a spacer region and, depending on the stringency of the similarity comparison, between 9 and 18 tandem copies of a 21 bp repeat within the CpG island. Sequence analysis of cDNAs from the MAGEL2-associated Unigene cluster Hs.141496 defined the limits of the cDNA cluster as nucleotide 71 651 at the 5′ end to nucleotide 73 911 at the 3′ end (numbering as in the PAC pDJ181P7 sequence). We performed RT–PCR on human brain RNA to demonstrate that no RT–PCR product could be amplified from the region 3′ to position 73 911, but RT–PCR products were obtained in the upstream region from position 69 618 to 69 919 (Fig. 2A) which we predict is in the 5′ half of the transcript. We were unable to amplify fragments that included the repeats (i.e. from position 69 919 to 71 651) using either reverse transcribed RNA or genomic DNA templates, suggesting that this region may be transcribed but is refractory to RT–PCR and cDNA cloning. In support of this hypothesis, the 5′ repeats are not represented among the cDNA clones we have identified to date. It is unlikely that there is additional 3′ untranslated sequence, since the human cDNAs derived from Unigene cluster Hs.141496 share a common region for their ends, and several have poly(A) tails suggesting that position 73 911 is the 3′ end of the transcript. Furthermore, the mouse cDNAs have a 3′-UTR of 427 bp, approximately the same size as the human 3′-UTR (see below). A series of repeats are located upstream of the predicted open reading frame with significant similarity to MAGEL2 (Fig. 2C) confirming and extending the conserved synteny of the murine and human gene clusters.

We then sequenced overlapping mouse ESTs and mouse genomic clones with high sequence similarity to MAGEL2 (Fig. 2B) and identified a predicted open reading frame with significant similarity to MAGEL2 (GenBank accession no. AF212306). A series of repeats are located upstream of the mouse open reading frame and consist of an 18 bp repeat reiterated 14.5 times. Contrary to a previous report (10), these repeats do not constitute a CpG island because they are either C/G-rich compared with genomic DNA nor do they contain a higher than average number of CpG dinucleotides. We also localized Magel2 to PAC clones containing Ndn were analyzed for the presence (+) or absence (−) of Zfp127 and Magel2 sequences by PCR with gene-specific primers.

The predicted proteins derived from MAGEL2 and its murine counterpart encode putative proteins of 529 and 525 amino acids, respectively (Fig. 3A). We predicted the first methionine to be the initiating methionine based on a superior Kozak consensus sequence (11) and significant amino acid similarity in the region between the first methionine and the second methionine. Amino acid similarities of the putative proteins derived from NDN, Ndn, MAGEL2 and Magel2 [presented incorrectly by Boccazio et al. (10)] are shown in Figure 3B. MAGEL2 contains an additional 188 amino acids at the N-terminus compared with NDN and does not have a proline-rich, acidic N-terminus like necdin does (12). We also compared the predicted amino acid sequence of MAGEL2 to other related human proteins using a phylogenetic analysis [PHYLIP (13)] (Fig. 3C). The region of highest similarity among MAGEL2, NDN, MAGE and MAGE-related proteins is in necdin ‘region 2’ (12). MAGEL2 has high similarity to the MAGF family of proteins in the region containing an antigenic nonapeptide implicated in
tumor rejection (14), whereas necdin is not similar to the MAGE proteins in the equivalent region.

Expression profiles of human and mouse MAGEL2

The cDNAs in MAGEL2 Unigene cluster Hs.141496 were derived from both adult and embryonic tissues. To investigate the expression pattern of MAGEL2, we examined a series of mouse and human tissues by northern blot analysis. MAGEL2 and Magel2 expression was detectable, albeit at low levels. A human cDNA probe detected a 4.5 kb transcript in fetal kidney, fetal brain, fetal liver, fetal lung and the putamen, temporal lobe, frontal lobe, occipital lobe, medulla, cerebral cortex and spinal cord of adult brain (Fig. 4A). This size corresponds to the predicted minimum size of 4.3 kb based on RT–PCR experiments (Fig. 2A). The mouse ESTs corresponding to Magel2 were derived only from late-stage embryos. Northern blot hybridization of mouse RNA revealed a moderately abundant 4.5 kb transcript in the late embryonic stages, E11, E15 and E17 (Fig. 4B). Whereas no expression was detected in adult tissues by northern blot, Magel2 RNA was predominantly detected in brain, with low levels of expression in other tissues by RT–PCR (10, and data not shown).

RNA in situ hybridization was performed to define the pattern of expression in mouse embryos. Consistent with our northern blot results, expression was detected in E9–E13 embryos (Fig. 5A–D). At E9, expression was found in the...
pharangeal region, the first branchial arch and a subset of somites at the lumbo-thoracic level. At E11, expression spreads over the somites in the caudal part of the embryo, and was detected in the dorsal part of the neural tube, and prominently in the mid- and hindbrain regions. High levels of expression were seen in the hypothalamus, the primary region of the brain involved in PWS, as well as in the telencephalon, medulla, cerebral cortex, mid-/hindbrain and spinal cord. Sites of Magel2 expression outside the neural tissue included the genital tubercule, a discrete region within the proximal part of the fore- and hindlimb buds, tongue and branchial arches in E9 embryos. The timing of Magel2 expression parallels that of neurogenesis, and suggests a role for MAGEL2 in the development of the nervous system. We then compared expression of Ndn and Magel2 in an E11 mouse embryo by whole-mount RNA in situ hybridization. Whereas Magel2 expression is highest in the ventricles and telencephalon at this stage, Ndn is expressed widely in the developing peripheral nervous system. We also detected Ndn expression in the proximal limb bud, which had not been previously described.

Imprinting of MAGEL2/Magel2

Using a single nucleotide polymorphism at position 73 614 that changes a restriction enzyme recognition site for Rsal, we determined that MAGEL2 expression is monoallelic in control human brain (data not shown). This information combined with the absence of expression of MAGEL2 in PWS brain suggests that loss of MAGEL2 expression in PWS is due to imprinting rather than being a direct consequence of the disease process. Magel2 is imprinted and not expressed from the paternal allele in newborn brain (10, and data not shown). To investigate imprinting at an earlier developmental stage, we detected Magel2 expression by RT–PCR in fibroblasts derived from parthenogenetic but not androgenic or primary mouse embryonic fibroblast control embryos (15), further suggesting paternal-only expression (Fig. 6A). Expression of Magel2 was also absent in brain from newborn mice carrying a paternally inherited deletion of the chromosome 7 PWS imprinting center.

Figure 4. Expression analysis of the necdin-like gene in embryonic and adult human and mouse tissues. (A) Northern blot of adult brain tissues (left) and of fetal tissues (right). The 4.5 kb MAGEL2 transcript is indicated by the arrow. (B) Northern blot of embryonic mouse tissues showing increasing expression of a 4.5 kb transcript (arrow) in later-stage embryos. Hybridization with control probes demonstrated approximately equal loading in each lane for (A) (ubiquitin control probe) and (B) (actin control).

Figure 5. Expression of Magel2 and Ndn in mid-gestation mouse embryos. Whole-mount RNA in situ hybridization with a Magel2 probe at (A) E9, (B) E13 (the embryo had been divided in two parts sagittally prior to processing), (C) lateral view at E11 and (D) dorsal view at E11. (E) Lateral views and (F) dorsal views of whole-mount RNA in situ hybridization with an Ndn probe (left) or a Magel2 probe (right) at E11. ba, first branchial arch; de, diencephalon; df, dorsal funiculus; gt, genital tubercle; hy, hypothalamic area; lb, proximo-caudal part of the limb bud (arrows); me, metencephalon; nt, dorsal part of the neural tube; ov, otic vesicle; p, pharangeal region; sg, spinal ganglion; sm, a subset of somites at the lumbo-thoracic level; sy, sympathetic ganglia; te, frontal part of telencephalon; tg, tongue; 4V, fourth ventricle; uRL, upper region (germinal trigone) of the rhombic lip.
human brain tested, although it shows only weak expression in spinal cord and cerebellum. This suggests a general role in neural differentiation or maintenance, rather than a specific role in one region of the brain. Low-level expression in non-neural tissues (e.g., in fetal kidney and lung) suggests either a non-neural role in these tissues, or expression in the peripheral nervous system components of these tissues. In the mouse, highest levels of expression occur in mid- to late-stage embryos with low expression in adult brain and trace expression in other tissues. Expression of Magel2 is very high in specific regions of the developing brain, including the hypothalamus. Hypothalamic deficiencies are noted in PWS and could be caused by the deficiency of a gene important in the development of the hypothalamus. Other correlations between the characteristics of PWS and Magel2 expression include small hands and feet (expression in the limb buds), articulation defects, dysmorphic mouth and philtrum, and thick viscous saliva (expression in the first branchial arch and tongue) and genital hypoplasia at birth (expression in the genitai tubercule). The expression patterns of Ndn and Magel2 partially overlap, since Ndn also shows strong expression in the central nervous system, including the hypothalamus, the proximal limb bud and the tongue (17). The persistence of Ndn expression in the adult contrasts with the primarily prenatal expression of Magel2.

MAGEL2 encodes a small protein of unknown function and has highest sequence similarity to necdin and the MAGE family of proteins. Necdin is simultaneously inactivated with the necdin-like gene in typical PWS, and we hypothesize that loss of both genes may be more detrimental than loss of necdin alone. Variable phenotypes have been reported among four independently created mouse strains bearing null mutations in Ndn alone. In two cases no phenotype was observed (18, and K. Yoshikawa et al., personal communication), whereas partial lethality was observed in two other cases (10,17). The differences in the phenotypic manifestation of necdin deletion may be due to differences in targeting strategies employed to eliminate the necdin protein (19). However, previously observed dependence of viability on strain background and on the presence or absence of the PGK-neo-cassette (17) suggests that one of the latter factors is likely to be responsible for variation in viability. Disruption of the imprinting center is completely lethal, due to loss of expression of multiple paternally expressed genes including Ndn (16) and Magel2 (this study). We conclude that the necdin-like gene (MAGEL2) may contribute to PWS when both necdin and necdin-like gene activity are disrupted. This hypothesis is based on the RNA expression pattern of Magel2 in the developing nervous system, which overlaps with that of necdin, also implicated in neuronal differentiation and PWS. Study of the function of the MAGEL2 and NDN proteins and further gene targeting experiments in the mouse will yield insight into the pathophysiology of PWS and the normal development of the brain.

Figure 6. Paternal origin of MAGEL2/Magel2 gene expression. (A) RT-PCR analysis of primary mouse embryonic fibroblasts. Cell types were androgenic embryonic fibroblasts (A), parthenogenetic embryonic fibroblasts (P), fertilized primary mouse embryonic fibroblasts (F) and, as a negative control, fertilized primary mouse embryonic fibroblasts with no reverse transcriptase (no RT). (B) RT-PCR analysis of Magel2 in PWS imprinting center deletion mice and littermate controls. Brain RNA from two different wild-type mice (wt) or two different mice heterozygous for the paternally inherited PWS imprinting center deletion mutation (mut) was used for RT-PCR with (+RT) or without (-RT) reverse transcriptase. (C) Northern blot analysis of necdin-deficient mice. No change in expression of the 4.5 kb transcript was seen in the embryonic day 19 fetuses carrying a paternally derived Ndn tm2Stw allele (lanes 1–3) or a wild-type Ndn allele (lanes 4–6).

DISCUSSION

We have independently identified and characterized the expression of the necdin-like gene, MAGEL2, and its mouse ortholog, Magel2. In human adult tissues, MAGEL2 has highest expression in brain, with wider expression in fetal tissues. MAGEL2 is equally expressed in many parts of the

MATERIALS AND METHODS

Genomic reagents

YAC clones 925C12 and 959G3 were obtained from Research Genetics (Huntsville, AL). Human PAC clone pDJ181P17 and mouse PAC RPCI-23 clones 480A23, 509A13 and 665K16 were obtained from the CGAT Genome Resource Facility
Hyperfilm (Amersham Pharmacia Biotech) was for 16 days at Ndn probes for (Hospital for Sick Children, Toronto, Ontario) by screening with 1818 Human Molecular Genetics, 2000, Vol. 9, No. 12.

The Nlg2F–Nlg9R PCR product was cloned into pCR-Blunt vector (Invitrogen, San Diego, CA) and used for both northern blot analysis of Ndn tm2Stw clearly hybridized with the radioactively labeled Nlg2F–Nlg9R transcript. A mouse embryo MTN blot (Clontech) was similarly hybridized with the radioactively labeled Nlg2F–Nlg9R probe. Two library (Stratagene, La Jolla, CA) were screened according to standard procedures (20) with the Nlg2F–Nlg9R probe. Two clones were clone names mj49f12, mr52b10 and mv40f06.

**Isolation of Magel2 mouse genomic clones**

The Nlg2F–Nlg9R PCR product was cloned into pCR-Blunt vector (Invitrogen, San Diego, CA) and used for both northern blot analysis and genomic library screening. Approximately 1.5 x 10^6 phage plaques of mouse 129SvEvTacIBR genomic library (Stratagene, La Jolla, CA) were screened according to standard procedures (20) with the Nlg2F–Nlg9R probe. Two clones were clone names mj49f12, mr52b10 and mv40f06. The sequencing data were assembled using GCG software package and verified by analyzing sequences of both strands.

**Northern blot expression studies.** A MAGEL2 535 bp probe generated from oligonucleotide primers NLG10F (5′-GACGCCATGATCTTTTCTA-3′) and NLG11R (5′-GCATCCC-CAGTCACTAGATTAGTAC-3′) was random prime [32P]dCTP labeled with the Random Primers DNA Labeling System (Life Technologies, Rockville, MD). The labeled probe was hybridized to Human Brain Multiple Tissue Northern Blot II (Clontech, Palo Alto, CA), and Human Fetal Multiple Tissue Northern Blot II (Clontech) in ExpressHyb solution according to manufacturer’s directions. The final wash was at 50°C in 0.1x SSC, 0.1% SDS twice for 20 min each time. Exposure to Hyperfilm (Amersham Pharmacia Biotech) was for 16 days at ~80°C. A control probe (ubiquitin) demonstrated approximately equal loading in all lanes. Primers NLG10F (5′-GACGCCATGATCTTTTCTA-3′) and NLG11R (5′-GCATCCC-CAGTCACTAGATTAGTAC-3′) were amplified with Pfu polymerase and DIG RNA labeling kit (Roche Biochemicals) from two linearized plasmid templates with the opposite insert orientation. Timed pregnant (day of plug detection is considered as E1) 129Sv females were sacrificed, conceptuses isolated in ice-cold phosphate-buffered saline, and processed for the whole-mount in situ hybridization as described (21). To facilitate the probe access, E13 embryos were cut in half sagitally before processing.

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Magel2 expression in uniparentally derived primary cell lines and in chromosome 7 imprinting center deletion mice

Total RNA isolated from androgenic, parthenogenetic and normally fertilized primary mouse embryonic fibroblast cell lines was reverse transcribed and subjected to PCR amplification with Nlg2F–Nlg9R primers. One-fifth of the reaction was electrophoresed on 1.8% agarose gel stained with ethidium bromide. RT–PCR amplification with the RT enzyme omitted served as a negative control. Total brain RNA (10 μg) from a chromosome 7 imprinting center deletion mouse was pretreated with DNase I (Gibco BRL, Gaithersburg, MD) and half of the reaction was subsequently used to synthesize first strand cDNA with superscript II reverse transcriptase (Gibco BRL) and random primers (Gibco BRL). The other half of the reaction was manipulated in parallel in the absence of RT. One-twentieth of the +RT or –RT reactions were used to program PCR reactions using primers Nlg2F–Nlg9R. Sequences of the Gapd primers were: 5′-TGACATCAGA-AGGTGAAG-3′ and 5′-TCTTGGAGGCCATGAGGCC-3′. PCR amplification conditions for Gapd were: 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and then 72°C for 30 s. The final cycle was followed by a 5 min extension period at 72°C.

RNA in situ hybridization to mouse embryos. A PCR-derived fragment, corresponding to the complete open reading frame of mouse Magel2 protein (positions 701–2288 as referred to in GenBank accession no. AF212306), and a PCR-derived fragment of Ndn open reading frame, were amplified with Pfu DNA polymerase (Stratagene) and cloned into pCR-Blunt vector (Invitrogen). The digoxigenin-labeled RNA sense- and antisense riboprobes were synthesized using T7 RNA polymerase and DIG RNA labeling kit (Roche Biochemicals) from two linearized plasmid templates with the opposite insert orientation. Timed pregnant (day of plug detection is considered as E1) 129Sv females were sacrificed, conceptuses isolated in ice-cold phosphate-buffered saline, and processed for the whole-mount in situ hybridization as described (21). To facilitate the probe access, E13 embryos were cut in half sagitally before processing.