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Complex regulation of multiple cytohesin-like genes in murine tissues and cells

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Abstract Three cytohesin-like cDNA molecules were isolated from murine ES cell derived embryoid bodies. The genomic structure of one of the three, CLM2, has been determined and transcriptional variants of each were isolated from a mouse brain cDNA library. The relative expression patterns of CLM1, 2, 3 and their transcriptional alternatives were determined by RT-PCR, nucleotide sequencing and RNA blotting. Their broad distribution and cell and tissue specific expression patterns suggest complex regulation during development and in the adult.

Key words: Cytohesin; Pleckstrin domain; Sec7 domain; Specific expression; Reverse transcription polymerase chain reaction

1. Introduction

Cellular 3-phosphoinositide phosphates (PtdIns) are generated by 3-phosphoinositide kinases (PI-3-kinases) activated by tyrosine kinase receptors and act as membrane associated second messengers in multiple biological systems. Signaling through PtdIns regulates membrane ruffling [19], membrane translocations [7], membrane trafficking [6] and cell adhesion [20]. Protein targets of PtdIns include isoforms of protein kinase C [18], the pleckstrin homology containing protein kinases c-Akt and Btk [5] and certain SH2 domains [15]. It is assumed that PtdIns are active in membrane localization of various signal transduction pathways.

A new intermediate was described by Klarlund et al. [8], who screened mouse fibroblast, adipocyte and brain libraries for proteins that bind PtdIns. A general receptor for phosphoinositides, GRP1, was isolated. GRP1 contains two conserved protein domains. The 5′ situated Sec7 domain is a mammalian homologue of the yeast Sec7 protein, a crucial component of protein transport between compartments of the Golgi apparatus [3]. Downstream of the Sec7 domain, GRP1 contains a pleckstrin homology domain that is responsible for PtdIns trisphosphate binding [8]. A similar protein, characterized by the two domain structure of GRP1, is B2-1. The B2-1 transcript was the first member of this gene family. Subtractive hybridization in natural killer cells and peripheral T cells was used for its isolation [11]. Additional homologues were isolated by affinity to the cytoplasmic tail of integrin β2 from a human T lymphoma cell line (cytohesin-1) by Kolanus et al. [9] and as nucleotide exchange factors (ARNO) for the small GTP binding protein, ARF, by Chardin et al. [1] and by Meacci et al. [13]. The quoted studies implicate cytohesin-like molecules as regulators of cell to matrix adhesion, protein sorting and vesicular transport in various mammalian cells.

We were interested in exploring the multiplicity and cell and tissue specific regulation of murine cytohesin-like molecules. Here we describe three cytohesin-like murine cDNAs with multiple transcriptional alternatives that are selectively expressed from the stem cells of early development to various tissues of the adult.

2. Materials and methods

2.1. Cell culture

R1 ES cells [14] were grown in Dulbecco's medium (DMEM) supplemented with 15% heat inactivated fetal calf serum, 2 mM glutamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mM 2-mercaptoethanol and leukemia inhibitory factor (LIF) at 37°C and 6.5% CO_2. ES cell derived embryoid bodies were grown according to Martin et al. [12]. Cells of the Lewis lung carcinoma and mouse melanoma (D122, A9, F10.9, and F1) lines were cultured in DMEM supplemented with 10% heat inactivated fetal calf serum.

2.2. Genomic and cDNA cloning

Total RNA was isolated with TRI-Reagent (Molecular Research Center). Polyadenylated mRNA (poly(A) mRNA) was purified, using the Poly(A)Tract mRNA isolation system (Promega). CLM1, 2 and 3 were amplified from embryoid body cultures (day 3) by the Titan One Tube RT-PCR System (Boehringer, Mannheim).

CLM1 was amplified by the primer pair 5′-GAGCTGGTGTCTGGCAGGAC-3′ (bases 3–22) and 5′-GAACTGGCAGAGAAGCCCTCT-3′ (bases 1361–1381) from rat Sec7A (GenBank accession number U83895, [17]). CLM2 was amplified from the same RNA source with the primers 5′-TGAAGGGAGAGTCTTTTCGGC-3′ (bases 31–51) and 5′-GATTTTCCAAACAGGGAACCAG-3′ (bases 1470–1491) from rat Sec7B (GenBank accession number U83896). The primer pair 5′-TCCCGGCTTTCAGCCCAGTC-3′ (bases 48–67) and 5′-ACTAGGACGGTGGCAGGAAAG-3′ (bases 1470–1490) from rat Sec7C (GenBank accession number U83897) was used to amplify CLM3. The RT-PCR products were cloned into the TA cloning vector, T-khs307, which was prepared by a modification of the Holton and Graham method [4]. Plasmid DNA was isolated by the alkali lysis method using the High Pure Plasmid isolation kit (Boehringer, Mannheim).

Transcriptional alternatives were isolated by PCR from a mouse brain cDNA library (Uni-ZAP XR cDNA library, Stratagene). The T3 sense primer (5′-AATTAACCCTCACTAAAGGG-3′) of the λ phage was the common member of the primer pairs. Specific antisense primers were synthesized from the CLM1 (5′-AGCAACTTCTGCTATCTCTTCC-3′, bases 167–188) and CLM2 (5′-CTCATAGCTTCACTTAGCTCTTC-3′, bases 247–269) sequences respectively. The amplified PCR products were cloned into the TA cloning vector.

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The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB013464, AB013465, AB013466, AB013467, AB013468, AB013469, and AB013470.

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DNA from R1 ES cells was used to establish the genomic map of CLM2. PCR primers were designed from the CLM2 cDNA. The primers were 5′-TGGCTTTCTTCCTGCGGACCT-3′ (bases 1–21, from CLM-2B), 5′-GTACAGGAAGCGGGCAATTTC-3′ (bases 470–490, from rat Sec7B) and 5′-CTTGGTGGAGCATGAACTTC-TA-3′ (bases 433–454, from rat Sec7B), 5′-GATTTTCCAAACAGG-GAACCAG-3′ (bases 1470–1491, from rat Sec7B). The PCR products were cloned into the TA cloning vector.

2.3. DNA sequencing and data analysis

Nucleotide sequences were determined on both strands of the insert DNA, using an automated DNA sequencer (Applied Biosystems) and DyeDeoxy terminator kits. Analysis of the predicted protein sequence was performed using BLAST [10].

2.4. Gene expression analysis

Tissues were collected from 13 week old random bred MF1 male mice. Expression of cytohesin genes in various mouse tissues, ES cells, ES cell derived embryoid bodies and tissue culture cell lines was analyzed by RT-PCR (30 cycles) with the following specific primers.

To detect CLM1-A transcripts, 5′-GAGCTGGTGTCTGGCAG-GAC-3′ (bases 3–22, from rat Sec7A) and 5′-GTTGTCAGTCA-GAATGAACCAG-3′ (bases 918–939 from rat Sec7A) were used as primers.

To detect CLM1-B, 5′-CGAGCTGACGTGGAGAACTG-3′ (bases 2–21, from CLM1-B) and 5′-CTGGGCAATGTCCTCA-CAAGTG-3′ (bases 351–372, from rat Sec7A) were used.

The CLM2-A alternative was detected by 5′-TGAAGGGA-GAGTCTTTTCGGC-3′ (bases 31–51, from rat Sec7B) and 5′-TCCGTAGTGTACTCAAAGTAGT-3′ (bases 1056–1077, from rat Sec7B). CLM2-B and C transcripts were detected by the same primer pair: 5′-TGGCTTTCTTCCTGCGGACCT-3′ (bases 1–21, from CLM2-B) and 5′-CTCATAGCTTCACTTAGCTCTTC-3′ (bases 247–269, from rat Sec7B).

CLM2-B and C differ by a deletion of 355 bp in the 5′ UTR (see Fig. 1 and text), thus these primers produce a 814 bp transcript for CLM2-B and a 459 bp transcript for CLM2-C.

Finally, CLM3-A was detected by 5′-TTCATCCTCACAGA-CAACTGCC-3′ (bases 973–994, from rat Sec7C) and 5′-TGGAGTCTGGATCTTGACTTTTC-3′ (bases 1319–1341, from rat Sec7C).

As a standard control, rat G3PDH was amplified by the primers GPD-S (5′-ACCACAGTCCATGCCATCAC-3′, bases 550–569, from rat G3PDH) and GPD-AS (5′-TCCACCACCCTGTTGCTGTA-3′, bases 982–1001, from rat G3PDH). RT-PCR products were electrophoresed on agarose and stained with ethidium bromide.

2.5. Northern and Southern blot analysis

RNA from various cell lines and tissues was electrophoresed on 1% agarose in MOPS-formaldehyde. Genomic DNA from ES cells was digested with restriction enzymes *Ssp*I, *Sca*I, *Pvu*II and *Hin*dIII (MBI). Restriction enzyme digests were electrophoresed on 0.8% agarose in 1×TBE buffer and blotted to Hybond N (Amersham). The transferred nucleic acids were linked to the membrane by UV cross-linking. DNA fragments (see text and Fig. 2A) were labeled with 5 μCi of [^{32}P]dATP. RNA blots were hybridized with the coding region of the CLM2-A cDNA (see text and Figs. 1 and 2) as probe. The blot was prehybridized for 2 h at 65°C in 5×SSPE, 5×Denhardt's solution, 0.5% SDS with 100 μg/ml of denatured salmon sperm DNA and it was washed 2×30 min in 0.2×SSC 0.1% SDS at 65°C.

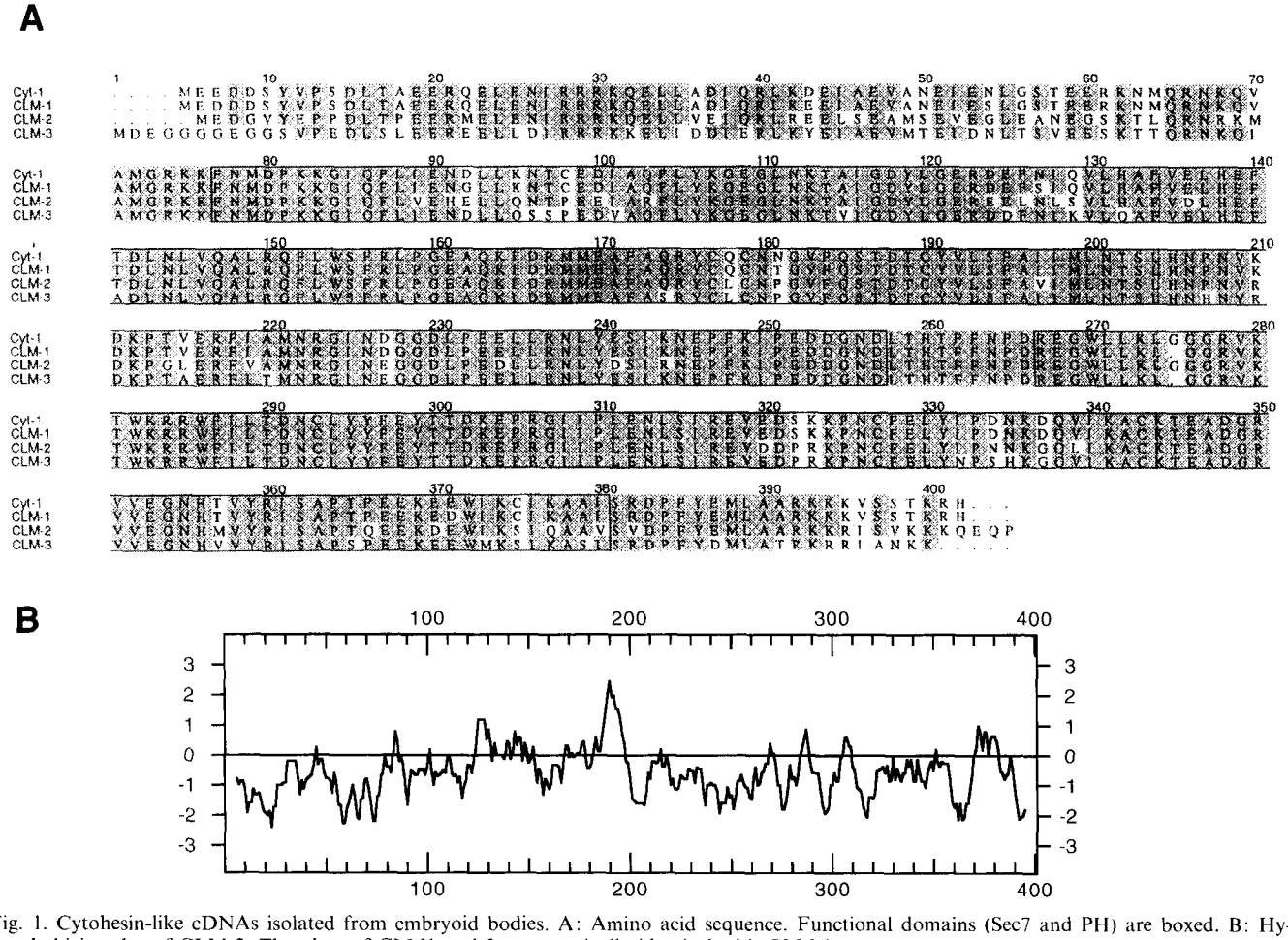

Fig. 1. Cytohesin-like cDNAs isolated from embryoid bodies. A: Amino acid sequence. Functional domains (Sec7 and PH) are boxed. B: Hydrophobicity plot of CLM-2. The plots of CLM1 and 3 are practically identical with CLM-2.

3. Results and discussion

3.1. Cytohesin-like cDNAs isolated from embryoid bodies

R1 ES cells were cultured on bacteriological tissue culture plates that do not allow adhesion and promote embryoid body differentiation [14]. RNA was isolated on the third day of culture, when visceral endoderm development had taken place and cavitation was at its beginning. RT-PCR amplification with probes derived from rat Sec7A,B and C cDNA sequences (see Section 2) yielded three distinct cytohesin-like murine (CLM) cDNA species (Fig. 1A).

The three sequences are highly homologous. Each contains an approximately 400 amino acid open reading frame with two distinguishable domains of homology. Between amino acid residues 77 and 257 the sequence is homologous to the mammalian Sec7-like domain, first described by Liu and Pohajdak [11]. Next between residues 267 and 380 a pleckstrin (PH) homology domain [16] can be distinguished. This Sec7 plus PH structure is characteristic for the human guanine nucleotide exchange factor ARNO [1,13] as well as for cytohesin-1 which was shown to interact with αLβ2 integrin [9], and for the general receptor for phosphoinositides, GRP1, isolated by Klarlund et al. from 3T3 fibroblast and mouse brain expression libraries [8].

Homology analysis revealed the relationship between the published amino acid sequences and the mouse sequences isolated by us from embryoid bodies. CLM1 was found to be similar to the human B2-1 cDNA [11]. CLM2 was highly similar to the human ARNO [1], whereas CLM3 was identical to the murine GRP1 gene [8]. The degree of amino acid homology between the transcripts was 80–90%, with 99% and 100% for the probably orthologous sequences. The highest homology between CLM1, 2 and 3 and between them and

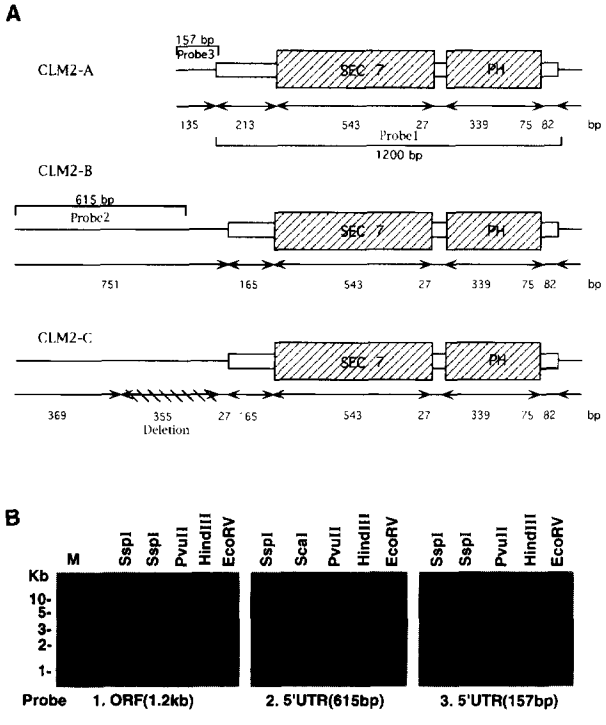

Fig. 2. Transcriptional alternatives of CLM-2. A: Comparative maps of CLM2A, B and C. B: Southern blot analysis of the transcriptional alternatives suggests that they derive from the same genomic locus (for probes see A).

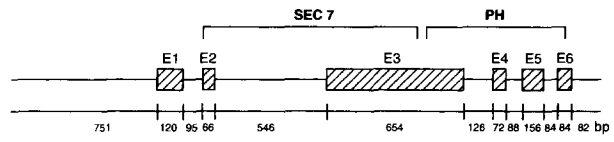

Fig. 3. Genomic map of CLM-2. Shaded boxes are coding exons.

other members of the family was observed in the Sec7 and PH coding domains, whereas the 5′ and 3′ coding sequences were considerably different, suggesting that the conserved sequences are the active domains of these proteins. CLM2 revealed a mostly hydrophilic structure (Fig. 1B), which was shared by CLM1 and 3, suggesting that cytohesins most likely are soluble molecules. Our observations indicate that cytohesin-like molecules may belong to a small family of related genes and that three distinct but related transcripts of this family are expressed together in differentiating ES cell derived embryoid bodies.

3.2. CLM2 is transcribed into multiple transcripts of a single genomic structure

Variant forms of the 5′ untranslated UTR of CLM2 were isolated from a mouse brain cDNA library (see Section 2). Three transcriptional alternatives were detected (Fig. 2A). CLM2-A had the shortest (135 bp) 5′ UTR, whereas the 5′ UTR of CLM2-B encompassed 751 bp; 355 bp of this 5′ extension was deleted in transcript CLM2-C.

Southern blot analysis with probes specific to the three transcriptional alternatives tested whether the three CLM2 alternatives derive from the same genomic structure. Probes from the appropriate 5′ UTRs revealed the same band pattern for all three (Fig. 2B), indicating that CLM2 is a single copy gene as also shown for the human B2-1 (cytohesin-1) gene [2].

To investigate the structure of the CLM2 locus, a PCR analysis was performed with 129 mouse DNA from R1 ES cells as template. Fig. 3 demonstrates that CLM2 is composed of six exons and introns with a 751 bp 5′ UTR. Interestingly the Sec7 and PH domains are not encoded by separate exons. The Sec7 homology is encoded by exons 2 and 3, whereas the PH homology is encoded by exons 3, 4, 5 and 6. Since the two

Table 1
Comparative analysis of CML gene expression by RT-PCR

Source (total RNA)	CLM1		CLM2			CLM3
	A	B	A	B	C	A
Tissues (adult)						
Spleen	+	+	+	+	–	+
Thymus	–	–	–	–	–	+
Salivary gland	+	–	+	+		+
Brain	+	+	+	+	+	+
Spinal cord	+	+	+	+	–	+
Heart	+	+	–	+	–	+
Muscle, skeletal	–	–	–	–		+
Stomach	+	–	+	+	–	+
Intestine	+	–	+	+	–	+
Lung	–	–	+	+	–	+
Kidney	+	+	+	+	–	+
Testis	+	–	+	+	+	+
Liver	+	–	+	+	–	+
Cell lines						
ES cells	+	–	+	+	–	+
Embryoid body	+	–	+	+	–	+
NIH 3T3	+	–	+	+	–	+
A20 (B cell)	+	–	+	+	–	+

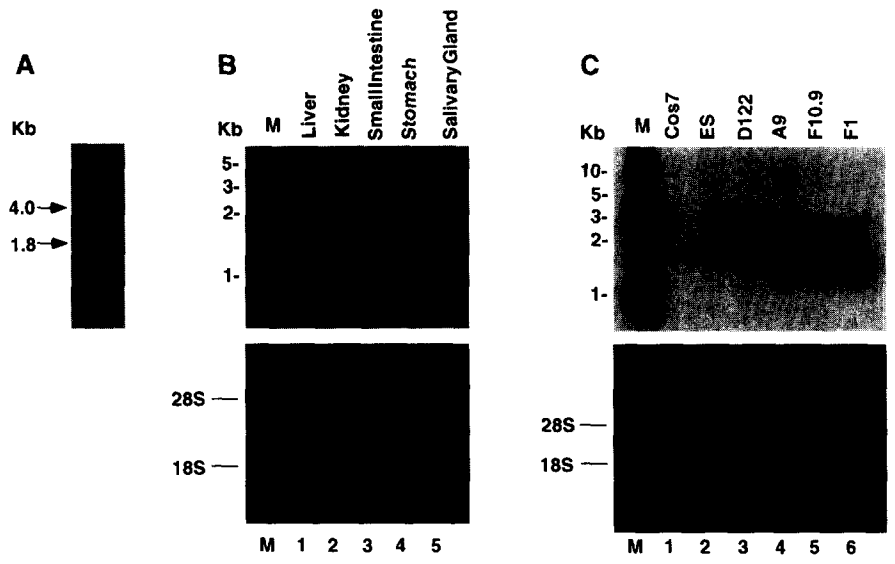

Fig. 4. RNA blot analysis of CLM2-A expression in different tissues. Lower panels shoe ethidium bromide staining of agarose gel. Probe: CLM2-A cDNA. A: Embryoid body, poly(A) rich RNA. B and C: Total RNA.

also occur separately in various genes [3,16] Sec7 and PH may have undergone considerable reshuffling during the evolution of CLM loci.

3.3. Cell and tissue specific expression of CLM1, 2 and 3

Poly(A)-rich RNA from differentiated embryoid bodies was hybridized at high stringency, with the coding region of CLM2-A as probe. Two transcripts were revealed, one approximately 1.8 kb and the other approximately 4.0 kb (Fig. 4A). Transcripts of similar size were detected throughout embryoid body differentiation (not shown) and also in mouse liver, kidney, organs of the alimentary canal and in the salivary gland (Fig. 4B) as well as in metastatic and non-metastatic tumor cells, whereas no expression could be detected in Cos cells (Fig. 4C).

Cell and tissue specific expression was studied by RT-PCR analysis using primers specific for CLM1-A and B, for CLM2-A, B and C as well as for CLM3-A. Investigation of numerous organs, cells and tissues revealed complex regulation of the multiple transcripts (Fig. 5). As shown in Table 1 the CLM1-A transcript was detected in all tissues and cell lines except for thymus, skeletal muscle and lung. This expression pattern was similar to those of CLM2-A and B, with small differences in their expression in heart and skeletal muscle Specific expression of CLM1-B was revealed in the central nervous system, heart and kidney, but not in other organs,

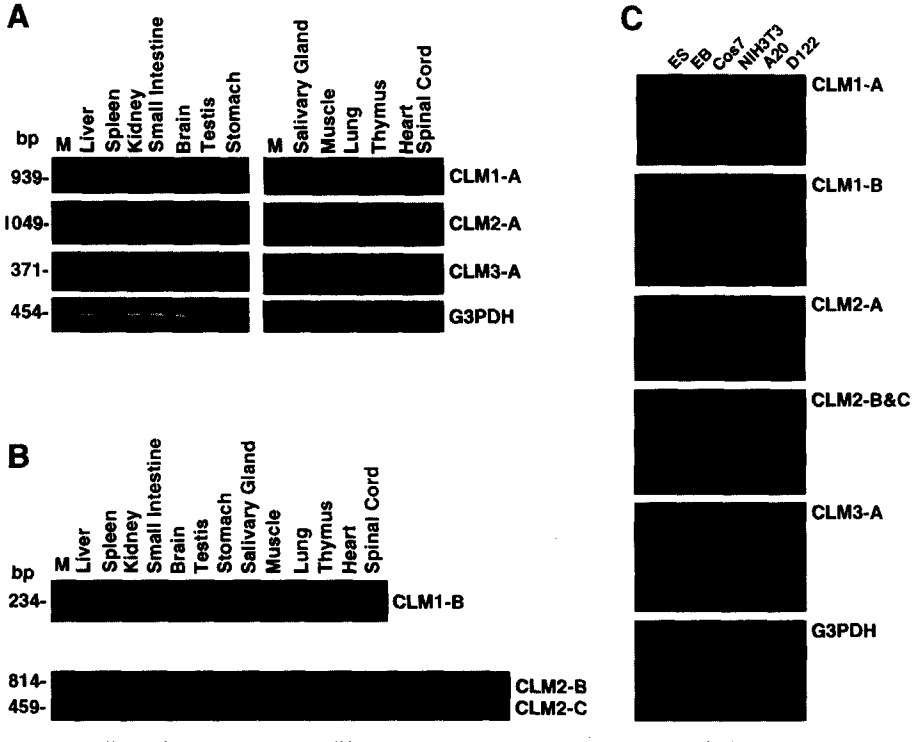

Fig. 5. Tissue and cell specific expression of different CLM transcripts; RT-PCR analysis. For primers, see Section 2.

or in the tumor cell lines. The other transcriptional alternative with restricted expression, CLM2-C, was expressed in brain and testis only. Finally, CLM3-A (GRP-1, [8]) was expressed in all tissues and cell lines.

Cytohesin-like molecules were first detected in lymphoid cells [11] and the effect of cytohesin-1 (CLM-1) on integrins was demonstrated in a T cell lymphoma line [9]. GRP1 (CLM-3) was isolated from multiple expression libraries. Chardin et al. also mention that ARNO, the human exchange factor for ARF, is generally expressed [1]. A similar conclusion was reached by Telemenakis et al. for the rat homologues of Sec7 [17], whereas our data suggest a complex, but specific expression pattern for these genes and their transcriptional alternatives.

Here we demonstrate that cytohesins form a small, related and highly homologous gene family. Three such molecules were isolated and the genomic structure of one, CLM2, was characterized. The murine CLM transcripts are highly homologous in the coding sequence of their Sec7 and PH domains. Each CLM has multiple transcriptional variants that are expressed in various organs with considerable relative specificity. Our data demonstrate that certain CLM transcriptional variants (CLM1-B and CLM2-C) display highly restricted expression patterns and with the exception of CLM3 (GRP1), the transcription of each has specific features. It follows from these observations that cytohesins and their transcriptional alternatives are under complex and multiple regulation in early embryonic cells and also in specific organs of the adult. We therefore speculate that members of this gene family and their transcriptional alternatives fulfill important specific functions in a complex spatial-temporal setting.

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References

[1] Chardin, P., Paris, S., Antonny, B., Robineau, S., Braud-Dufour, S., Jackson, C.L. and Chabre, M. (1996) Nature 384, 481–484.
[2] Dixon, B., Mansour, M. and Pohajdak, B. (1993) Cytogenet. Cell Genet. 63, 42–44.
[3] Franzusoff, A. and Schekman, R. (1989) EMBO J. 8, 2695–2702.
[4] Holton, T.A. and Graham, M.W. (1991) Nucleic Acids Res. 19, 1156.
[5] James, J.R., Downes, C.P., Gigg, R., Grove, S.J., Holmes, A.B. and Alessi, D.R. (1996) Biochem. J. 315, 709–713.
[6] Joly, M., Kazlauskas, A., Fay, F.S. and Corvera, S. (1994) Science 263, 684–687.
[7] Kanai, F., Ito, K., Todaka, M., Hayashi, H., Kamohara, S., Ishii, K., Okada, T., Hazeki, O., Ui, M. and Ebina, Y. (1993) Biochem. Biophys. Res. Commun. 195, 762–768.
[8] Klarlund, J.K., Guilherme, A., Holik, J.J., Virbasius, J.V., Chawla, A. and Czech, M.P. (1997) Science 275, 1927–1930.
[9] Kolanus, W., Nagel, W., Schiller, B., Zeitlemann, L., Godar, S., Stockinger, H. and Seed, B. (1996) Cell 86, 233–242.
[10] Lipman, D.J., Altschul, S.F. and Kaceciogiu, J.D. (1989) Proc. Natl. Acad. Sci. USA 86, 4412–4415.
[11] Liu, L. and Pohajdak, B. (1992) Biochim. Biophys. Acta 1132, 75–78.
[12] Martin, G.R., Wiley, L.M. and Damjanov, I. (1977) Dev. Biol. 61, 230–244.
[13] Meacci, E., Tsai, S.-C., Adamik, R., Moss, J. and Vaughan, M. (1997) Proc. Natl. Acad. Sci. USA 94, 1745–1748.
[14] Nagy, A. and Rossant, J. (1993) in: Gene Targeting: A Practical Approach (Joyner, Ed.), Oxford University Press, Oxford.
[15] Rameh, L.E., Chen, C.S. and Cantley, L.C. (1995) Cell 83, 821–830.
[16] Shaw, G. (1996) BioEssays 18, 35–46.
[17] Telemenakis, I., Benseler, F., Stenius, K., Sudhof, T. and Brose, N. (1997) Eur. J. Cell Biol. 74, 143–149.
[18] Toker, A., Meyer, M., Reddy, K.K., Falck, J.R., Aneja, R., Aneja, S., Parra, A., Burns, D.J., Ballas, L.M. and Cantley, L.C. (1994) J. Biol. Chem. 269, 32358–32367.
[19] Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., CLaesson-Welsh, L. and Stephens, L. (1994) Curr. Biol. 4, 385–393.
[20] Zell, T., Hunt, S.W., Mobley, J.L., Finkelstein, L.D. and Shimizu, Y. (1996) J. Immunol. 156, 883–886.