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Molecular Identification of Two Novel Munc-18 Isoforms Expressed in Non-neuronal Tissues*

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Munc-18, also known as n-Sec1 or rbSec1, is a syntaxin-binding protein thought to play a role in regulating synaptic vesicle exocytosis. Although a gene family of syntaxins has been identified, only a limited subset bind to Munc-18. This implicates the existence of other mammalian Munc-18 homologues that may be involved in a range of vesicle transport reactions. The purpose of the present study was to identify other members of the Munc-18 family by cDNA cloning. Three distinct Munc-18 isoforms, Munc-18a, previously identified in neuronal tissue, and two novel isoforms, Munc-18b and Munc-18c, were isolated from a 3T3-L1 adipocyte cDNA library by screening with a rat brain Munc-18 DNA probe. Munc-18a is identical to Munc-18 and by Northern analysis is expressed predominantly in brain and to a lesser extent in testis and 3T3-L1 cells. Munc-18b is 62% identical to Munc-18 at the amino acid level and is expressed in testis, intestine, kidney, rat adipose tissue, and 3T3-L1 cells. Munc-18c is 51% identical to Munc-18 and is ubiquitously expressed. It is likely, based on these findings, that unique Munc-18/syntaxin interactions may play an important role in generating a combinatorial mechanism for the regulation of vesicle transport in mammalian cells.

Identifying the molecular factors that regulate vesicle transport and fusion in eukaryotic cells has been the subject of intense investigation (1, 2), particularly in the mammalian synapse. Here it has been demonstrated that many of the proteins that regulate synaptic vesicle exocytosis are similar to those observed in other cell types, including yeast, thus enabling the formulation of unifying models to explain all vesicle transport reactions (3).

Recent biochemical studies have identified a number of multiprotein intermediates (SNARES)[1] that regulate the specificity of synaptic vesicle exocytosis (reviewed in Ref. 4). A fusion protein complex, consisting of *N*-ethylmaleimide-sensitive factor and soluble *N*-ethylmaleimide-sensitive factor attachment proteins, interact with three proteins isolated from brain: synaptobrevin or vesicle-associated membrane protein, a synaptic vesicle membrane protein; and syntaxin and the synaptosomal-associated protein-25 (SNAP-25), both found on the presynaptic plasma membrane. The identification of this protein complex supports the recent SNARE hypothesis (3), which implies that the specificity of membrane fusion is regulated by the specific binding of the donor vesicle (via the V-SNARE or synaptobrevin protein) with the acceptor membrane (via a T-SNARE or syntaxin). This basic template for molecular recognition and regulation of membrane fusion appears to be a general mechanism, since both T-SNARE and V-SNARE components belong to large gene families. In mammalian cells six syntaxin homologues have been cloned which exhibit a broad tissue distribution as well as variations in subcellular location (5). Two distinct synaptobrevins, 1 and 2 (6), have been identified in synaptic vesicles, whereas cellubrevin, a synaptobrevin homologue, is targeted to recycling endosomes (7). Other members of these two gene families have also been described in yeast. The yeast syntaxin homologues, Sed5p (8), Sso1p, Sso2p (9), and Pep12p (10), together with a variety of synaptobrevin homologues, including Bet1p (11, 12), Bos1p (13), SNC1, and SNC2 (14) are thought to coordinate specific membrane trafficking events throughout the secretory pathway.

Another family of proteins believed to participate in this complex show homology to Sec1p. The Sec1p family of proteins includes Sly1 (15), Slp1/Vps33 (16), and Sec1 (17, 18), which act at many different stages along the secretory pathway in yeast. Studies in mammalian cells, however, have so far only identified one Sec1p family member, Munc-18/n-Sec1/rbSec1 (19–21), giving rise to the question as to whether other Sec1p-like proteins exist in mammalian cells. Munc-18 itself was identified as a mammalian syntaxin-binding protein. Although there is no functional data concerning the role of Munc-18 in vesicle transport, a highly related protein from *Caenorhabditis elegans* unc-18, has been identified and mutations in this gene product result in accumulation of acetylcholine containing secretory vesicles as well as abnormalities in the development of the *C. elegans* nervous system (22, 23). Thus, given the likelihood of additional mammalian Sec1p-like proteins and their potentially important role in membrane trafficking events, we have identified and characterized further members of this gene family to more closely understand the regulation of vesicle transport.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). Radioactive nucleotides and nylon membranes (Hybond-N$^+$) were from Amersham (Aylesbury, United Kingdom). Oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer. All chemicals were high purity commercial grades. 3T3-L1 fibroblasts, obtained from the American Type Tissue Culture Center, were cultured and differentiated into adipocytes as described previously (24).

cDNA Cloning and Sequence Analysis of Munc-18 Isoforms—Two

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U19520 (munc-18b gene) and U19521 (munc-18c gene).

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[1] The abbreviations used are: SNAREs, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors; V-SNARE, vesicle membrane SNARE; T-SNARE, target membrane SNARE; Munc-18, mammalian homologue of unc-18; kb, kilobase(s); bp, base pair(s).

oligonucleotide primers corresponding to the 5′ (TGCTCTAGAAGAACGCCATGGCCCCCATTGG; sense primer) and 3′ (TGCTCTAGATTAACTGCTTATTTCTTCGTCTGTTTTATTCAG; antisense primer) ends of the rat brain Munc-18 cDNA (19) were synthesized and used to obtain a full length Munc-18 DNA fragment by reverse transcriptase-polymerase chain reaction from rat brain RNA. Rat brain RNA was isolated by the guanidine isothiocyanate procedure (25). The polymerase chain reaction cycling profile was 94 °C for 20 s, 55 °C for 20 s, 72 °C for 2 min, repeated for 35 cycles. The authenticity of the amplified DNA fragment was verified by restriction mapping. The polymerase chain reaction product was isolated from a 1% agarose gel, radiolabeled with random hexamer primers (Promega Corp., Madison, WI) and used to screen a random-primed 3T3-L1 adipocyte cDNA library constructed in λ ZAP II, kindly provided by Dr. F. Fiedorek, University of North Carolina. A total of 250,000 plaques were screened. From 35 positives, isolated after sequential purifications, three distinct cDNA classes were identified. Clones were subcloned into pBluescript II SK− (Stratagene, La Jolla, CA) and sequenced manually using Sequenase version 2.0 (U. S. Biochemical Corp.) or by automated DNA sequencing (Applied Biosystems Inc., model 373A). Both strands of DNA for the entire Munc-18c cDNA and 97% of the Munc-18b cDNA were sequenced utilizing T3, T7, and gene-specific oligonucleotide primers as well as the Erase-a-base nested deletion kit (Promega). The remaining 5′ sequence of Munc-18b was obtained using the 5′-Amplifinder rapid amplification of cDNA ends kit (Clontech, Palo Alto, CA), as per manufacturer's instructions. 3T3-L1 adipocyte poly(A)$^+$ RNA (2 μg) was reverse-transcribed and then primed with oligonucleotide P1 (GCTGCTTTGTAGGTGAAGGTTGGTGTTCCC). A nested gene-specific primer P2 (CGCGGATCCCGTGGGACTCAGCAAATAAATTGCCTCC) was used in conjunction with this procedure. The deduced amino acid sequences of Munc-18b and Munc-18c were aligned with rat brain Munc-18a (19–21) and the *C. elegans* gene product unc-18 (23) using the computer program Clustal V (26).

RNA Blot Analysis—Total RNA was isolated from rat tissues, 3T3-L1 fibroblasts, and adipocytes by the guanidine isothiocyanate procedure (25). 3T3-L1 fibroblast and adipocyte poly(A)$^+$ was obtained using the Pharmacia mRNA purification kit (Pharmacia, Uppsala, Sweden). RNA was electrophoresed using a 1% formaldehyde-agarose resolving gel and transferred to a nylon membrane. The blot was sequentially probed with Munc-18a, Munc-18b, and Munc-18c DNA fragments labeled with [α-^{32}P]dCTP by random priming. The hybridization conditions were: 50% formamide, 5 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution, 1% SDS, and 100 μg/ml denatured herring sperm DNA at 42 °C for 16 h. The blot was washed with 1 × SSC and 0.1% SDS at 50 °C. The RNA blots were also probed with DNA coding for glyceraldehyde-3-phosphate dehydrogenase for normalization of results. The conditions for hybridization of the RNA blot for each of the three Munc-18 DNA probes was individually determined such that they did not cross-hybridize.

RESULTS

To examine the hypothesis that there is a large gene family of mammalian Munc-18 isoforms, a 3T3-L1 adipocyte cDNA library was screened with a 1.8-kb rat brain Munc-18 DNA fragment. Thirty-five positive clones were isolated, and 30 were characterized by DNA sequencing. Three distinct cDNA classes were identified, referred to here as Munc-18a, Munc-18b, and Munc-18c. Munc-18a was identical to Munc-18/n-Sec1/rbSec1 (19–21) as determined by sequencing the 5′ 340 nucleotides of a full-length clone (M5A) and by restriction mapping. Three of the 30 characterized clones were assigned to this group. Munc-18b was the most abundant isoform isolated from the library (22 out of 30 clones), whereas the remaining five clones fell into the third class, Munc-18c. Both Munc-18b and Munc-18c represent novel Munc-18 isoforms. The inserts from two separate Munc-18b clones (M1B and M3A) were completely sequenced on both strands and were both found to be missing 5′ ends, as was the case for all the remaining clones in this class. The 5′-coding region was obtained using a 5′ rapid amplification of cDNA ends procedure (see "Experimental Procedures"). A 270-bp DNA fragment was amplified by this technique and then subcloned into Bluescript, sequenced, and found to contain a start codon with a consensus Kozak sequence (27) followed by 18 bp of novel sequence and 200 bp that were identical to that of the 5′ end of M1B. The entire open reading frame of Munc-18b is 1,779 bp encoding a protein of 593 amino acids with a predicted M_r of 66,357 and pI of 6.72 (Fig. 1). A 2.5-kb Munc-18c full-length clone (M2I) was sequenced in both directions and found to contain a 1,776-bp open reading frame encoding a protein of 592 amino acids with a calculated M_r of 67,942 and a pI of 7.96 (Fig. 2). None of these deduced amino acid sequences showed any evidence of a transmembrane region. The percentage identity between the three Munc-18 isoforms at the nucleotide level is 54–64%.

The amino acid sequences of the three Munc-18 3T3-L1 isoforms exhibit substantial similarity along their entire length to the *C. elegans* gene product, unc-18 (Fig. 3). Munc-18b and Munc-18c showed 62 and 51% amino acid identity, respectively, compared with Munc-18/n-Sec1/rbSec1, referred to here as Munc-18a (Fig. 3, Table I). We have adopted a similar nomenclature to Hata *et al.* (19) to refer to these different isoforms rather than that used by others (20, 21), since all of the clones isolated from mammalian cells showed a higher degree of amino acid identity to the *C. elegans* gene product, unc-18, than to the yeast homologue, Sec1p (Table I). As shown in Table I, Munc-18a is most similar to the *Drosophila* homologue, Ropp (65%) and *C. elegans* unc-18 (59%), whereas Munc-18b is increasingly less similar to Ropp (54%) and unc-18 (53%) with Munc-18c being the least identical to Ropp (44%) and unc-18 (43%). All three adipocyte Munc-18 homologues displayed much lower identities (17–27%) to Sec1p, Sly1p, and Slp1p (Table I). The predicted secondary structures of Munc-18a, Munc-18b, and Munc-18c were all very similar (results not shown).

The tissue distribution of Munc-18a, Munc-18b, and Munc-18c was studied by Northern blot analysis and was found to be unique for each isoform (Fig. 4*A*). In agreement with previous studies (19–21), Munc-18a had a transcript size of 3.8 kb and was expressed predominantly in rat brain, but lower levels were also detected in testis. Munc-18b was expressed at highest levels in rat testis, and lower levels of expression were detected in intestine, kidney, and epididymal fat pad. A major Munc-18b transcript of 2.3 kb and a minor transcript of 3.2 kb were detected in each of these tissues (Fig. 4*A*). The molecular basis and significance of the two transcripts remains to be determined. Munc-18c (transcript size: 3.0 kb) was expressed ubiquitously in liver, kidney, intestine, testis, heart, skeletal muscle, brain, and epididymal fat. A second Munc-18c transcript of 1.7 kb was also detected in rat testis. Each of the Munc-18 isoforms was expressed in 3T3-L1 fibroblasts and adipocytes, consistent with the fact that these clones were isolated from a 3T3-L1 adipocyte cDNA library. The expression of Munc-18a decreased following differentiation into adipocytes, whereas increased mRNA levels of both Munc-18b and Munc-18c were observed in 3T3-L1 adipocytes compared with the undifferentiated fibroblasts (Fig. 4*B*).

DISCUSSION

In this study we have identified two novel Munc-18 isoforms which exhibit broad and distinct tissue distributions and which are highly homologous to the previously described neural isoform, Munc-18/n-Sec1/rbSec1 (19–21). We have referred to these gene products as: Munc-18a, which is the neural-specific protein; Munc-18b, found in testis, kidney, intestine, and adipose tissue; and the ubiquitously expressed Munc-18c. Previous studies in a variety of organisms have ascribed an important role to the Munc-18/Sec1p gene product in the regulation of vesicle transport. In the mammalian synapse Munc-18a has been shown to bind to the presynaptic membrane protein syntaxin (19–21). Furthermore, a genetic interaction between Sec1p and two syntaxin homologues, Sso1 and Sso2, has been found in yeast (9). In view of the putative role of syntaxin in the

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gtgggctattcctcaggggaag ATG GCG CCC TTG GGG CTG AAG GCG GTG GTA GGG GAA AAA ATT TTA AGC GGC GTC ATC CGG    82
                       met ala pro leu gly leu lys ala val val gly glu lys ile leu ser gly val ile arg    20

AGC GTT AAG AAG GAT GGC GAG TGG AAG GTC CTC ATC ATG GAT CAC CCG AGC ATG CGA ATC TTG TCA TCC TGT TGT AAA   160
ser val lys lys asp gly glu trp lys val leu ile met asp his pro ser met arg ile leu ser ser cys cys lys    46

ATG TCA GAT ATC CTG GCG GAG GGC ATC ACC ATC GTG GAA GAC ATC AAC AAA CGG AGA GAA CCC ATT CCC AGC TTG GAG   238
met ser asp ile leu ala glu gly ile thr ile val glu asp ile asn lys arg arg glu pro ile pro ser leu glu    72

GCA ATT TAT TTG CTG AGT CCC ACG GAG AAG TCG GTT CAG GCC CTG ATT GCG GAC TTC CAG GGA ACA CCA ACC TTC ACC   316
ala ile tyr leu leu ser pro thr glu lys ser val gln ala leu ile ala asp phe gln gly thr pro thr phe thr    98

TAC AAA GCA GCC CAT ATC TTC TTC ACT GAC ACA TGC CCT GAG CCC TTG TTC AGT GAA CTG GGC CGC TCT CGC CTG GCG   394
tyr lys ala ala his ile phe phe thr asp thr cys pro glu pro leu phe ser glu leu gly arg ser arg leu ala   124

AAG GCG GTG AAG ACA TTG AAA GAG ATC CAC CTT GCC TTC CTC CCC TAT GAG GCC CAG GTG TTC TCT CTG GAT GCC CCA   472
lys ala val lys thr leu lys glu ile his leu ala phe leu pro tyr glu ala gln val phe ser leu asp ala pro   150

CAT AGC ACC TAC AAC CTT TAC TGT CCA TTC CGA GCA GGG GAG CGG GGG CGG CAG CTC GAT GCA CTG GCC CAG CAG ATA   550
his ser thr tyr asn leu tyr cys pro phe arg ala gly glu arg gly arg gln leu asp ala leu ala gln gln ile   176

GCC ACA CTG TGT GCC ACT CTT CAG GAA TAC CCA TCC ATC CGA TAC CGC AAG GGT CCA GAA GAC ACA GCC CAG CTG GCC   628
ala thr leu cys ala thr leu gln glu tyr pro ser ile arg tyr arg lys gly pro glu asp thr ala gln leu ala   202

CAT GCT GTC CTG GCC AAG CTG AAC GCC TTC AAG GCA GAC ACT CCC AGT CTG GGC GAG GGC CCA GAG AAA ACA CGC TCA   706
his ala val leu ala lys leu asn ala phe lys ala asp thr pro ser leu gly glu gly pro glu lys thr arg ser   228

CAG CTA CTC ATA ATG GAC CGG GCA GCA GAC CCC GTG TCC CCA CTA TTA CAC GAA CTC ACG TTC CAA GCC ATG GCC TAT   784
gln leu leu ile met asp arg ala ala asp pro val ser pro leu leu his glu leu thr phe gln ala met ala tyr   254

GAT CTT CTG GAC ATC GAA CAG GAC ACA TAC AGG TAT GAG ACC ACA GGG CTG AGT GAG TCC CGG GAG AAG GCT GTC CTC   862
asp leu leu asp ile glu gln asp thr tyr arg tyr glu thr thr gly leu ser glu ser arg glu lys ala val leu   280

CTG GAT GAA GAT GAT GAC CTG TGG GTG GAA CTT CGG CAC ATG CAC ATA GCA GAT GTT TCC AAG AAG GTC ACA GAA CTC   940
leu asp glu asp asp asp leu trp val glu leu arg his met his ile ala asp val ser lys lys val thr glu leu   306

CTG AAG ACA TTC TGT GAG AGT AAG AGG CTG ACC ACA GAC AAG GCC AAC ATC AAA GAC CTG TCC CAC ATC CTG AAA AAG  1018
leu lys thr phe cys glu ser lys arg leu thr thr asp lys ala asn ile lys asp leu ser his ile leu lys lys   332

ATG CCA CAG TAT CAG AAG GAG CTG AAC AAG TAC TCT ACG CAC CTG CAT TTG GCA GAT GAC TGC ATG AAG CAT TTC AAG  1096
met pro gln tyr gln lys glu leu asn lys tyr ser thr his leu his leu ala asp asp cys met lys his phe lys   358

GGC TCG GTG GAG AAG CTG TGC AGT GTG GAG CAG GAC CTG GCC ATG GGC TCT GAT GCA GAG GGT GAG AAG ATC AAG GAC  1174
gly ser val glu lys leu cys ser val glu gln asp leu ala met gly ser asp ala glu gly glu lys ile lys asp   384

GCC ATG AAG CTG ATT GTC CCG GTG CTG CTG GAT GCC TCG GTG CCA CCC TAC GAC AAG ATC CGG GTT CTG TTG CTC TAC  1252
ala met lys leu ile val pro val leu leu asp ala ser val pro pro tyr asp lys ile arg val leu leu leu tyr   410

ATC CTT CTG CGG AAT GGG GTG AGT GAG GAG AAC CTG GCC AAG CTG ATC CAG CAT GCC AAC GTG CAG TCG TAC AGC AGC  1330
ile leu leu arg asn gly val ser glu glu asn leu ala lys leu ile gln his ala asn val gln ser tyr ser ser   436

CTC ATC CGG AAT CTG GAG CAG CTG GGC GGC ACT GTC ACC AAC TCC GCG GGC TCA GGG ACC TCT AGC CGG CTG GAG CGG  1408
leu ile arg asn leu glu gln leu gly gly thr val thr asn ser ala gly ser gly thr ser ser arg leu glu arg   462

AGA GAG CGC ATG GAG CCC ACC TAC CAG CTG TCT CGC TGG TCC CCA GTC ATC AAG GAT GTG ATG GAG GAT GTG GTG GAG  1486
arg glu arg met glu pro thr tyr gln leu ser arg trp ser pro val ile lys asp val met glu asp val val glu   488

GAC CGG CTA GAC CGA AAG CTG TGG CCC TTT GTG TCT GAC CCT GCC CCT GTG CCT AGC TCC CAG GCA GCT GTC AGC GCT  1564
asp arg leu asp arg lys leu trp pro phe val ser asp pro ala pro val pro ser ser gln ala ala val ser ala   514

CGC TTT GGC CAC TGG CAC AAA AAC AAA GCT GGA GTA GAG GCC CGG GCT GGG CCT AGG CTC ATC GTG TAC ATT GTG GGT  1642
arg phe gly his trp his lys asn lys ala gly val glu ala arg ala gly pro arg leu ile val tyr ile val gly   540

GGT GTC GCC ATG TCG GAA ATG AGG GCT GCC TAT GAG GTG ACC AGG GCC ACT GAG GGC AAG TGG GAG GTG CTC ATA GGC  1720
gly val ala met ser glu met arg ala ala tyr glu val thr arg ala thr glu gly lys trp glu val leu ile gly   566

TCT TCT CAC ATC CTT ACT CCA ACC CGC TTC CTC GAT GAC CTC AAG ACA CTG GAT CAG AAG CTG GAG GGT GTG GCC CTG  1798
ser ser his ile leu thr pro thr arg phe leu asp asp leu lys thr leu asp gln lys leu glu gly val ala leu   592

CCC TGA cacagccccttgcctctgccctc                                                                        1827
Pro ***                                                                                                593
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FIG. 1. **Complete nucleotide sequence of 3T3-L1 Munc-18b cDNA and deduced amino acid sequence of the protein.** *Capital letters* indicate the coding sequence, whereas *lowercase letters* signify the 5′- and 3′-noncoding sequences. Amino acid residues are denoted by the standard three-letter code below the nucleotide sequence, and the sequences are numbered on the *right*.

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                                                                                gaattcggcacgagcgtacacacctagcccttccagtgccacatctcgtccgt    54

agctctggattcttagacttcagttggacatttgtttttctctttagttgtatttcctgggtttttgtgatgatcaatggactttaaagctccacggctggggg   156

aag ATG GCG CCG CCG GTA TCG GAG CGG GGA CTG AAG AGC GTC GTG TGG CGG AAG ATA AAA ACA GCC GTG TTC GAT GAC   234
    met ala pro pro val ser glu arg gly leu lys ser val val trp arg lys ile lys thr ala val phe asp asp    25

TGC CGG AAA GAA GGC GAA TGG AAG ATA ATG CTG TTA GAT GAG TTT ACC ACC AAA CTT TTG TCG TCA TGC TGC AAA ATG   312
cys arg lys glu gly glu trp lys ile met leu leu asp glu phe thr thr lys leu leu ser ser cys cys lys met    51

ACA GAC CTT CTA GAG GAG GGC ATA ACT GTT ATA GAG AAT ATT TAT AAG AAT CGT GAA CCT GTC AGA CAA ATG AAA GCT   390
thr asp leu leu glu glu gly ile thr val ile glu asn ile tyr lys asn arg glu pro val arg gln met lys ala    77

CTT TAT TTC ATC TCT CCA ACA CCA AAA TCT GTA GAT TGT TTC TTG AGA GAT TTT GGA AGT AAA TCT GAG AAA AAA TAC   468
leu tyr phe ile ser pro thr pro lys ser val asp cys phe leu arg asp phe gly ser lys ser glu lys lys tyr   103

AAA GCA GCG TAT ATA TAC TTC ACT GAC TTT TGT CCT GAC AGT CTC TTT AAC AAG ATT AAA GCA TCT TGC TCC AAG TCA   546
lys ala ala tyr ile tyr phe thr asp phe cys pro asp ser leu phe asn lys ile lys ala ser cys ser lys ser   129

ATA AGA AGA TGT AAG GAA ATA AAC ATT TCC TTC ATT CCA CAG GAA TCT CAG GTT TAT ACT CTT GAT GTA CCG GAC GCA   624
ile arg arg cys lys glu ile asn ile ser phe ile pro gln glu ser gln val tyr thr leu asp val pro asp ala   155

TTC TAT TAC TGT TAC AGT CCA GAC CCT AGT AAC GCC AGC AGG AAA GAA GTG GTC ATG GAG GCA ATG GCT GAG CAG ATT   702
phe tyr tyr cys tyr ser pro asp pro ser asn ala ser arg lys glu val val met glu ala met ala glu gln ile   181

GTG ACA GTG TGT GCC ACT CTG GAT GAA AAC CCT GGA GTG AGG TAC AAG AGT AAA CCT CTA GAT AAT GCC AGT AAG CTT   780
val thr val cys ala thr leu asp glu asn pro gly val arg tyr lys ser lys pro leu asp asn ala ser lys leu   207

GCA CAG CTG GTT GAG AAA AAG CTT GAA GAC TAC TAC AAA ATT GAT GAA AAA GGC CTA ATA AAG GGT AAA ACT CAG TCC   858
ala gln leu val glu lys lys leu glu asp tyr tyr lys ile asp glu lys gly leu ile lys gly lys thr gln ser   233

CAG CTC TTA ATA ATT GAC CGT GGC TTT GAC CCT GTG TCC ACT GTC CTG CAT GAA CTG ACC TTT CAG GCA ATG GCA TAT   936
gln leu leu ile ile asp arg gly phe asp pro val ser thr val leu his glu leu thr phe gln ala met ala tyr   259

GAT CTA CTA CCA ATT GAG AAT GAT ACA TAC AAG TAC AAA ACA GAT GGA AAA GAG AAG GAG GCA GTT CTT GAA GAA GAC  1014
asp leu leu pro ile glu asn asp thr tyr lys tyr lys thr asp gly lys glu lys glu ala val leu glu glu asp   285

GAC GAC CTG TGG GTG CGG GTT CGA CAC CGG CAC ATC GCG GTT GTG TTG GAG GAA ATT CCA AAG CTT ATG AAG GAA ATT  1092
asp asp leu trp val arg val arg his arg his ile ala val val leu glu glu ile pro lys leu met lys glu ile   311

TCA TCA ACA AAG AAA GCT ACA GAG GGG AAG ACA TCA CTT AGC GCT CTT ACC CAG CTG ATG AAA AAG ATG CCG CAC TTC  1170
ser ser thr lys lys ala thr glu gly lys thr ser leu ser ala leu thr gln leu met lys lys met pro his phe   337

CGA AAG CAG ATC TCG AAG CAA GTA GTC CAT CTT AAC TTA GCT GAA GAC TGC ATG AAT AAG TTT AAG CTG AAT ATT GAG  1248
arg lys gln ile ser lys gln val val his leu asn leu ala glu asp cys met asn lys phe lys leu asn ile glu   363

AAG CTC TGC AAA ACT GAG CAG GAC CTG GCA CTT GGA ACA GAT GCT GAA GGC CAG CGG GTG AAG GAC TCC ATG CTG GTG  1326
lys leu cys lys thr glu gln asp leu ala leu gly thr asp ala glu gly gln arg val lys asp ser met leu val   389

CTC CTC CCA GTG CTG CTC AAC AAA AAC CAT GAC AAC TGC GAC AAA ATA CGG GCA GTC CTG CTC TAC ATC TTC GGG ATT  1404
leu leu pro val leu leu asn lys asn his asp asn cys asp lys ile arg ala val leu leu tyr ile phe gly ile   415

AAT GGA ACC ACT GAA GAA AAT CTG GAC AGA CTG ATC CAC AAT GTA AAG ATA GAA GAT GAT AGT GAT ATG ATT CGT AAC  1482
asn gly thr thr glu glu asn leu asp arg leu ile his asn val lys ile glu asp asp ser asp met ile arg asn   441

TGG AGC CAC CTT GGT GTT CCC ATT GTT CCC CCA TCC CAG CAA GCC AAA CCA CTG AGA AAG GAT CGG TCT GCA GAA GAG  1560
trp ser his leu gly val pro ile val pro pro ser gln gln ala lys pro leu arg lys asp arg ser ala glu glu   467

ACT TTT CAG CTT TCT CGA TGG ACA CCT TTT ATC AAA GAT ATC ATG GAG GAT GCC ATT GAT AAT AGA TTA GAT TCC AAA  1638
thr phe gln leu ser arg trp thr pro phe ile lys asp ile met glu asp ala ile asp asn arg leu asp ser lys   493

GAG TGG CCG TAT TGT TCC CGG TGC CCA GCA GTG TGG AAT GGC TCT GGA GCT GTG AGT GCT CGC CAG AAA CCC AGA ACT  1716
glu trp pro tyr cys ser arg cys pro ala val trp asn gly ser gly ala val ser ala arg gln lys pro arg thr   519

AAC TAC TTA GAG CTG GAC CGG AAA AAT GGG TCA AGG CTG ATT ATT TTT GTA ATT GGA GGA ATT ACG TAC TCT GAG ATG  1794
asn tyr leu glu leu asp arg lys asn gly ser arg leu ile ile phe val ile gly gly ile thr tyr ser glu met   545

CGG TGT GCT TAT GAA GTT TCC CAG GCA CAT AAA TCC TGT GAG GTT ATT ATT GGT TCC ACA CAT ATT TTA ACA CCC AGA  1872
arg cys ala tyr glu val ser gln ala his lys ser cys glu val ile ile gly ser thr his ile leu thr pro arg   571

AAG CTC CTG GAT GAT ATA AAA ATG CTG AAT AAA TCA AAG GAT AAA GTT TCC TTT AAG GAT GAG TAA ctttttatggttgtc  1953
lys leu leu asp asp ile lys met leu asn lys ser lys asp lys val ser phe lys asp glu ***                    592

gtttagaggttttggxtaatacgatcggctacagcaaatgttgcttgttgtaatttaagcggggtaaatagggtatggagtaatg                  2038
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FIG. 2. **Complete nucleotide sequence of 3T3-L1 Munc-18c cDNA and deduced amino acid sequence of the protein.** *Capital letters* indicate the coding sequence, whereas *lowercase letters* signify the 5′- and 3′-noncoding sequences. Amino acid residues are denoted by the standard three-letter code below the nucleotide sequence, and the sequences are numbered on the *right*.

formation of a vesicle docking complex, it has been suggested that members of the Munc-18/Sec1p family may play a proofreading function in vesicle docking/fusion reactions (20).

The SNARE hypothesis (3) proposes that the specificity of different vesicle fusion reactions is determined by specific proteins in both the donor and acceptor membrane compartments.

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Munc-18a    MAPI----GLKAVVGEKIMHDVIKKVKK---KGEWKVLVVDQLSMRMLSSCCKMTDIMTE  53
Munc-18b    MAPL----GLKAVVGEKILSGVIRSVKK---DGEWKVLIMDHPSMRILSSCCKMSDILAE  53
Munc-18c    MAPPVSERGLKSVVWRKIKTAVFDDCRK---EGEWKIMLLDEFTTKLLSSCCKMTDLLEE  57
 unc-18     M-------SLKQIVGHKLLNDVIRPLKKGDGRSAWNVLIVDTLAMRMLSSCCKMHNIMEE  53
            *       .** .*  *.   *.   .*    . *.....*  . ..******* ... *

Munc-18a    GITIVEDINKRREPLPSLEAVYLITPSEKSVHSLISDFKDPPTAKYRAAHVFFTDSCPDA 113
Munc-18b    GITIVEDINKRREPIPSLEAIYLLSPTEKSVQALIADFQGTPTFTYKAAHIFFTDTCPEP 113
Munc-18c    GITVIENIYKNREPVRQMKALYFISPTPKSVDCFLRDFGSKSEKKYKAAYIYFTDFCPDS 117
 unc-18     GITIVEDLNKRREPLPTLEAIYLIAPTAESIDKLIQDY--CARNLYKCAHVFFTEACSDQ 111
            ***..*.. * ***.  . *.*...*.  *.. .. *.   .   *. * ..**. *..

Munc-18a    LFNELVKSRAAKVIKTLTEINIAFLPYESQVYSLDSADSFQSFYSPH--KAQMKNPILER 171
Munc-18b    LFSELGRSRLAKAVKTLKEIHLAFLPYEAQVFSLDAPHSTYNLYCPF--RAGERGRQLDA 171
Munc-18c    LFNKI-KASCSKSIRRCKEINISFIPQESQVYTLDVPDAFYYCYSPDPSNASRKEVVMEA 176
 unc-18     LFSTLSKSAAARFIKTLKEINIAFTPYESQVFNLDSPDTFFLYYNAQ--KQGGLTSNLER 169
            **. . ..  .. ..   **...* * *.**. ** ...    * .          ..

Munc-18a    LAEQIATLCATLKEYPAVRYRGE-YKDNALLAQLIQDKLDAY-KADDPTMGEGPDKARSQ 229
Munc-18b    LAQQIATLCATLQEYPSIRYRKG-PEDTAQLAHAVLAKLNAF-KADTPSLGEGPEKTRSQ 229
Munc-18c    MAEQIVTVCATLDENPGVRYKSKPLDNASKLAQLVEKKLEDYYKIDEKGLIKG--KTQSQ 234
 unc-18     IAEQIATVCATLGEYPSLRYRAD-FERNVELGHLVEQKLDAY-KADDPSMGEGADKARSQ 227
             *.** *.**** * *..**.         *.. .  **. . * *   .  *  *..**

Munc-18a    LLILDRGFDPSSPVLHELTFQAMSYDLLPIENDVYKYETSGIGEARVKEVLLDEDDDLWI 289
Munc-18b    LLIMDRAADPVSPLLHELTFQAMAYDLLDIEQDTYRYETTGLSESREKAVLLDEDDDLWV 289
Munc-18c    LLIIDRGFDPVSTVLHELTFQAMAYDLLPIENDTYKYKTDG----KEKEAVLEEDDDLWV 290
 unc-18     LIIIDRGYDAITPLLHELTLQAMCYDLLGIENDVYKYETGGSDENLEKEVLLDENDDLWV 287
            *.*.**. *.  . .*****.*** **** **.* *.* * *      *  .*.*.****.

Munc-18a    ALRHKHIAEVSQEVTRSLKDFSSSK-RMNTGEKTTMRDLSQMLKKMPQYQKELSKYSTHL 348
Munc-18b    ELRHMHIADVSKKVTELLKTFCESK-RLTT-DKANIKDLSHILKKMPQYQKELNKYSTHL 347
Munc-18c    RVRHRHIAVVLEEIPKLMKEISSTK--KATEGKTSLSALTQLMKKMPHFRKQISKQVVHL 348
 unc-18     EMRHKHIAVVSQEVTKNLKKFSESKGNKGTMDSKSIKDLSMLIKRMPQHKKELNKFSTHI 347
             .** *** *   .   .*. .*    *     .  *. ..*.**. .*...*   *.

Munc-18a    HLAEDCMKHYQGTVDKLCRVEQDLAMGTDAEGEKIKDPMRAIVPILLDANVSTYDKIRII 408
Munc-18b    HLADDCMKHFKGSVEKLCSVEQDLAMGSDAEGEKIKDAMKLIVPVLLDASVPPYDKIRVL 407
Munc-18c    NLAEDCMNKFKLNIEKLCKTEQDLALGTDAEGQRVKDSMLVLLPVLLNKNHDNCDKIRAV 408
 unc-18     SLAEECMKQYQQGVDKLCKVEQDLSTGIDAEGERVRDAMKLMVPLLIDPAVRCEDRLRLI 407
             **..**. ..  ..***  ****. * ****....*.*  ..*.*..      *..* .

Munc-18a    LLYIFLKNGITEENLNKLIQHAQIPPEDSEIITNMAHLGVPIVTDSTLRRRSKPERKERI 468
Munc-18b    LLYILLRNGVSEENLAKLIQHANVQSYSS-LIRNLEQLGGTVTNSAGSGTSSRLERRERM 466
Munc-18c    LLYIFGINGTTEENLDRLIHNVKIE-DDSDMIRNWSHLGVPIVPPS---QQAKPLRKDRS 464
 unc-18     LLYILSKNGITDENLNKLLQHANISMADKETITNAAYLGLNIVTDTGRKKTWTPTKKERP 467
            ****.  ** ..*** .*... ..       * *   **  .  .         ...*

Munc-18a    SEQTYQLSRWTPIIKDIMEDTIEDKLDTKHYPYISTRSSASFSTTAVSARYGHWHKNKAP 528
Munc-18b    -EPTYQLSRWSPVIKDVMEDVVEDRLDRKLWPFVSDPAPVPSSQAAVSARFGHWHKNKAG 525
Munc-18c    AEETFQLSRWTPFIKDIMEDAIDNRLDSKEWPYCSRCPAVWNGSGAVSAR--QKPRTNYL 522
 unc-18     HEQVYQSSRWVPVIKDIIEDAIDERLDTKHFPFLAGRQVNQGYRAPASARYGQWHKERGQ 527
             *  .* *** * ***..** ....** *   . .          . ***  .  .

Munc-18a    -GEYRSGPRLIIFILGGVSLNEMRCAYEVTQA-NGKWEVLIGSTHILTPQKLLDTLKKLN 586
Munc-18b    -VEARAGPRLIVYIVGGVAMSEMRAAYEVTRATEGKWEVLIGSSHILTPTRFLDDLKTLD 584
Munc-18c    ELDRKNGSRLIIFVIGGITYSEMRCAYEVSQAHKSC-EVIIGSTHILTPRKLLDDIKMLN 581
 unc-18     QSNYRSGPRLIIYIIGGVTFSEMRACYEVT-AARKPWEVVIGSDRIITPDKFLTNLRDLN 586
              . . *.***....**.. .***  ***. *     **.*** .*.** ..*  .. *.

Munc-18a    KTDEEIS-S-- 594
Munc-18b    QKLEGVALP-- 593
Munc-18c    KSKDKVSFKDE 592
 unc-18     KPRD----I-- 591
            . .
```

FIG. 3. **Comparison of the deduced amino acid sequences of Munc isoforms.** Three different Munc-18 clones were isolated from a 3T3-L1 adipocyte cDNA expression library. Munc-18a was partially sequenced (see *underlined region*) and was found to be identical to a rat brain isoform previously referred to as Munc-18/nSec1/rbSec1 (19–21). Munc-18b and Munc-18c are novel cDNAs. Also included in the alignment is a *C. elegans* gene product, unc-18 (GenBank accession number S66176). Amino acids are shown in the single-letter code and numbered on the *right*. Amino acids that are identical between all four homologues are denoted by an *asterisk*, whereas conserved substitutions are indicated with a *dot*. *Gaps* introduced to generate this alignment are represented by *dashes*. Sequences were aligned using the computer program Clustal V.

TABLE I

Sequence similarities between Munc-18 homologues

The amino acid identities between mammalian (Munc-18s), yeast (Sec1p, Slp1p, Sly1p), *C. elegans* (unc-18), and *Drosophila* (Ropp) homologues.

	Munc-18a	Munc-18b	Munc-18c	Ropp	Unc-18	Sec1p	Sly1p	Slp1p
Munc-18a		62	51	65	59	27	22	20
Munc-18b	62		46	54	53	26	21	17
Munc-18c	51	46		44	43	23	22	17

In the case of the mammalian synapse, these include syntaxin, synaptobrevin, synaptosomal-associated protein-25 (SNAP-25), and small molecular weight GTP-binding proteins of the Rab family (reviewed in Ref. 4). Each of these proteins belongs to a large gene family, the individual members of which display differences in tissue expression and intracellular location. Another difference is that both genetic studies in yeast and biochemical studies in the mammalian synapse have revealed a particular specificity in the interaction between different members of these gene families. For instance, synaptobrevin or vesicle-associated membrane protein 1 and 2 bind to syntaxin 1A and 4 but not to syntaxin 2 and 3 (28). On the basis of such

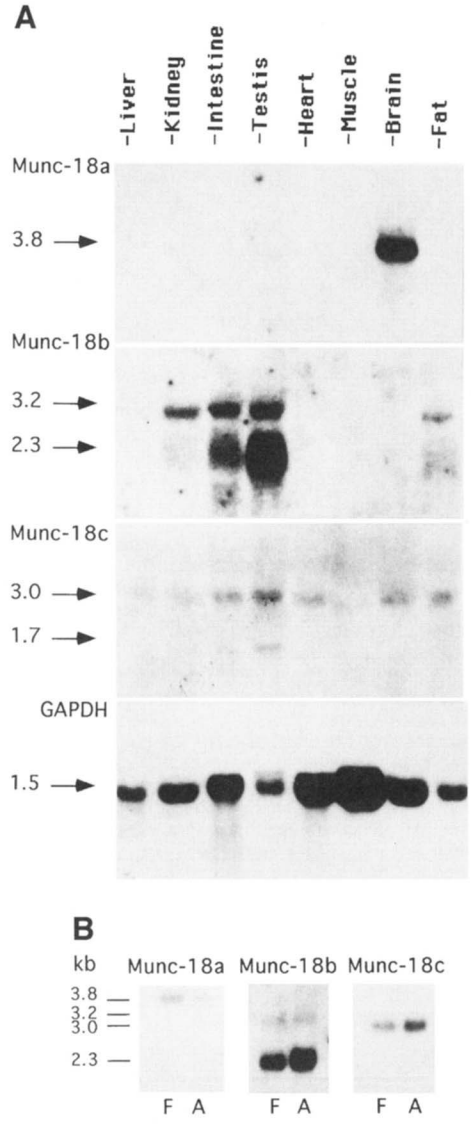

FIG. 4. **RNA blot analysis of 3T3-L1 Munc-18 isoform mRNAs in various rat tissues and 3T3-L1 fibroblasts and adipocytes.** Total RNA from eight different rat tissues and poly(A)$^+$ RNA from 3T3-L1 fibroblasts and adipocytes were hybridized with α-^{32}P-labeled probes derived from the three different Munc-18 isoforms (Munc-18a, Munc-18b, and Munc-18c) as well as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as described under "Experimental Procedures." *A*, expression of Munc-18a, Munc-18b, Munc-18c, and glyceraldehyde-3-phosphate dehydrogenase mRNAs in rat tissues. *B*, expression of Munc-18a, Munc-18b, and Munc-18c mRNAs in 3T3-L1 fibroblasts (*F*) and adipocytes (*A*). The size (in kilobases) of the major transcripts are indicated at the *left*.

studies it has been suggested that the specificity of vesicle transport may be regulated by a combinatorial mechanism. We propose on the basis of the findings in the present study that different Munc-18 isoforms are also involved in regulating distinct vesicular transport steps, thus elaborating the fidelity of a combinatorial mechanism of sorting.

Two lines of evidence predicted the existence of multiple homologues of Munc-18 in mammalian cells. First, three distinct yeast genes have been identified that are involved in discrete vesicle transport events. These include Sec1 (17), which is involved in post-Golgi secretion, Sly1 (15), in endoplasmic reticulum to Golgi transport, and Slp1 (16), that has a function in vacuolar trafficking. Second, the neural homologue of Sec1, referred to here as Munc-18a, binds to syntaxin 1A, 2, and 3 but not to syntaxin 4 (20). Furthermore, the expression of Munc-18a is primarily confined to neural tissue (19–21; Fig. 4*A*), whereas members of the syntaxin family display a broad tissue distribution (5).

Based on the amino acid homology between Munc-18a, Munc-18b, and Munc-18c as well as their tissue distributions, it is likely that they may be involved in a wide array of vesicle transport steps. Munc-18b showed the highest amino acid sequence identity to Munc-18a (62%, Table I). The relatively limited tissue distribution of Munc-18b implies that it may be involved in more specialized vesicle trafficking events common to testis, intestine, and kidney. The tissue distribution of Munc-18b does not correlate with that of any of the known mammalian syntaxin isoforms (5). This raises the possibility that a Munc-18b-specific syntaxin remains to be identified. However, the neural-specific isoform, Munc-18a, interacts with multiple syntaxin's (20), both neural and non-neural, and so it is conceivable that different Munc-18/syntaxin combinations may occur *in vivo*, depending upon the native expression of these proteins in a particular cell type. Munc-18c exhibited a broad tissue distribution implicating its involvement in a more constitutive vesicle transport event that is common to all cells. Consistent with this, Munc-18c showed the least identity to Munc-18a, Ropp, and unc-18 (43–51%, Table I), which have all been implicated in regulated exocytosis in more specialized secretory cells. In order to define the role of these new Munc-18 isoforms in vesicle transport, it will be necessary to determine the intracellular location of Munc-18b and Munc-18c using isoform-specific antibodies and to study the specificity of the interaction between different members of the syntaxin and Munc-18 gene families.

The identification of a mammalian Munc-18 gene family provides further insight into our understanding of vesicle transport regulation. These proteins are likely to play an important role in vesicle docking and/or fusion and should be considered as an integral component of the SNARE complex. Future studies will be required to map the specific interactions between different syntaxin's and Munc-18 isoforms in order to define their role in vesicle transport. It is also possible that other members of this gene family exist, which are either not expressed at a significant level in 3T3-L1 cells or are less homologous than the three Munc-18 proteins described here.

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