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Disruption of the Yeast *N*-Myristoyl Transferase Gene Causes Recessive Lethality

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The structural gene for *N*-myristoyl transferase (*NMT1*) has been cloned from the budding yeast *Saccharomyces cerevisiae*. The gene encodes a polypeptide of 455 amino acids (M_r = 52,837) that has no identifiable significant primary sequence homology with any protein in currently available databases. Overexpression of NMT activity was achieved by means of the yeast episomal plasmid YEp24 without obvious effects on growth kinetics, cell morphology, or acylprotein metabolic labeling patterns. Insertional mutagenesis of the *NMT1* locus on yeast chromosome XII caused recessive lethality, indicating that this protein acyltransferase activity is necessary for vegetative cell growth.

N-MYRISTOYL TRANSFERASE (NMT) catalyzes the co-translational (*1*) attachment of myristic acid (C14:0) via an amide linkage to the NH_2-terminal glycine residues of certain cellular and viral proteins. Myristoyl proteins are targeted to several cellular locations, including the plasma membrane, cytosol, and endoplasmic reticulum, and are involved in such diverse processes as protein phosphorylation, signal transduction, and oncogenesis (*2*). Viral myristoyl proteins include the VP4 and VP2 capsid components of some picornaviruses and papovaviruses, respectively, the *gag* polyprotein precursors of many mammalian retroviruses, and the human immunodeficiency virus *nef* (*3′-orf*) gene product (*3*). Abolishing myristoylation of certain oncoproteins and retroviral *gag* proteins can have profound biological effects. For example, site-directed mutagenesis of the Gly^1 residue of $pp60^{vsrc}$ to Ala^1 or Glu^1 prevents myristate attachment, interferes with the protein's stable association with plasma membranes, and blocks its ability to transform cells (*4*). Analogous mutagenesis of the *gag* polyprotein precursors of some mammalian retroviruses suggests that the myristoyl moiety is critical for their association with plasma membranes and proper assembly of mature virions (*5*). However, since the various myristoyl proteins are distributed widely among both membrane and cytosolic compartments, myristoylation cannot be solely responsible for targeting a specific protein to a particular intracellular location.

NMT has been purified from *Saccharomyces cerevisiae* and its fatty acyl coenzyme A (CoA) thioester and peptide substrate specificities examined in vitro (*6*). The enzyme shows remarkable specificity for myristoyl CoA, excluding other fatty acids based on chain length rather than hydrophobicity, and has an absolute requirement for NH_2-terminal glycine. Binding of the fatty acyl CoA appears to influence subsequent binding of peptide substrates. Comparative kinetic analyses indicate that the substrate specificities of NMT are highly conserved among yeast, plant, and mammalian cells. The gene for NMT has now been cloned from *S. cerevisiae* to determine the structure of its primary translation product, and to take advantage of the genetic manipulability of yeast to probe the role of NMT in cell growth.

NMT was purified to apparent homogeneity from *S. cerevisiae* strain BJ405 as previously described (*6, 7*), except that the final fast protein liquid chromatography (FPLC) step was deleted and a microbore C4 high-performance liquid chromatography (HPLC) step substituted. The principal peak contained a single 55-kD protein, as defined by silver staining of an SDS-polyacrylamide gel. NMT activity coeluted with this polypeptide as measured by an in vitro assay (*8*). Sequence analysis of the purified 55-kD protein revealed that its NH_2-terminus was blocked. The protein was therefore digested with trypsin and the resulting peptides separated by C18 reversed-phase HPLC and subjected to Edman degradation (*9*). After consulting yeast codon usage tables (*10*), we synthesized nondegenerate oligonucleotides (45 and 48 bases long) on the basis of the sequences of two of these peptides.

Blot hybridization of total yeast DNA indicated that both oligonucleotides hybridized to 5.4-kb Hind III and 5.7-kb Pst I fragments. Hind III– or Pst I–digested genomic DNA fragments were fractionated on agarose gels to enrich for the appropriate sized pieces and introduced into pUC13 DNA. The resulting two libraries were screened for bacterial colonies that hybridized specifically with both oligonucleotides.

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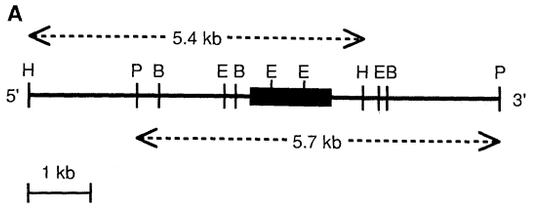

Fig. 1. Primary nucleotide and amino acid sequence of the *NMT1* structural gene of *S. cerevisiae*. (**A**) Restriction map of the *NMT1* locus. Total cellular DNA was prepared from strain AB972 (*7, 25*), digested with Hind III or Pst I, and fractionated through 0.8% agarose gels. DNA fragments from 4.5 kb to 6.5 kb were obtained from each digestion by band interception with Schleicher & Schuell NA-45 anion exchange paper (according to the manufacturer's specifications) and cloned into pUC13. *Escherichia coli* strain DH5α was transformed with the recombinant plasmids and colonies screened with two synthetic oligonucleotides: 5′-TTCGGTCCAGGTGACGGTTTCTTGAACTTCTACTTGTTCAACTACAGA-3′ and 5′-ATCACCGACTTCTTCTCCTTCTACTCCTTGCCATTCACCATCTTG-3′. Cloned genomic DNA sequences having 5.4-kb Hind III or 5.7-kb Pst I fragments are shown (arrows). Each reacted with both oligonucleotides. B, Bam HI; E, Eco RI; P, Pst I; and H, Hind III. The thick solid bar represents *NMT1* coding DNA. (**B**) Primary structure of *NMT1*. The nucleotide sequence of both coding and noncoding strands was determined by the dideoxy chain termination method with modified T7 DNA polymerase (U.S. Biochemical Corp.). Nested deletions of M13 mp18 and mp19 subclones containing the 2.1-kb Bam HI–Hind III double oligo positive restriction fragment were used for these reactions (*28*). Underlined amino acids were confirmed by Edman degradation of NMT tryptic peptides. Underlined bold amino acids correspond to the two peptides from which the oligos were made. Small vertical arrows indicate the major sites of transcription initiation determined by avian myeloblastosis virus reverse transcriptase (Seikagaku America) extension of the primer 5′-CAATAAATTCTCTAATTTTTTCGC-3′ (horizontal arrow above nucleotides 204 to 227) after its annealing to total cellular RNA prepared from strain YM2061 (*7, 29*). Potential TATA transcriptional regulatory elements are boxed. A consensus polyadenylation signal beginning at nucleotide 1693 is underlined twice. The Eco RV site used for disruption of the *NMT1* gene (Fig. 2) at nucleotide 1018 is enclosed in square brackets.

Overlapping DNAs representing approximately 7.5 kb of the yeast genome were isolated (Fig 1A). Nucleotide sequence analysis of a 2.1-kb Bam HI–Hind III fragment that reacted with both oligonucleotides revealed a single open reading frame spanning 1,365 nucleotides. The open reading frame specifies a protein of 455 amino acids with a calculated M_r of 52,837 (Fig 1B). This figure agrees with previous size estimates of yeast NMT (*6*). All six tryptic fragments from which unambiguous sequence information was obtained are precisely represented in different regions of the deduced translation product. Among these is a peptide that occurs between the first two in-frame NH_2-terminal methionine residues, allowing us to identify the initiator AUG codon. No "consensus" sequence for protein *N*-myristoylation (*6*) is present at or near this NH_2-terminus, suggesting that the blocking

Table 1. Introduction of multiple copies of the *NMT1* gene per cell produces an increase in NMT specific activity. Constructs made in YEp24: in pBB110 a small portion of the *Tc*^{r} gene of YEp24 is replaced with a 2.1-kb Bam HI–Sal I restriction fragment containing the entire *NMT1* coding region plus 115 nucleotides of 5′ nontranscribed sequence; pBB113 has an additional 1.15-kb of 5′ upstream sequence cloned in the correct orientation into the Bam HI site of pBB110. All *NMT1* DNAs used in these constructs were obtained from a recombinant plasmid having 5.4 kb of the *NMT1* locus (cloned as a Hind III fragment into pUC13, Fig. 1A). Lithium acetate–treated cells of strain YM2061 (*7*) were transformed to Ura$^+$ with pBB110, pBB113, or YEp24 DNA (*19*). To determine *NMT1* gene copy number per cell of the YM2061 transformants, total yeast cellular DNA was prepared (*25*) from mid–log phase cells grown in synthetic complete glucose media (*19*) lacking uracil (except YM2061) to select for the plasmids. The DNA samples were digested with Bam HI or Bam HI + Sal I, blotted to Gene Screen Plus (Du Pont), and probed with a ^{32}P-labeled Bam HI–Pvu II fragment of pBB110 containing the *NMT1* gene and some vector sequences. Reactive band sizes on the blots confirmed the presence of the plasmids as episomal species. *NMT1* copy number in pBB110 and pBB113 transformants is expressed as the ratio of vector:endogenous *NMT1*-derived band intensities as measured by laser densitometeric scanning of filter autoradiographs. Three independent experiments were performed in which portions (0.5, 1.0, 2.0, 4.0, and 8.0 μl) of crude lysates from the indicated strains (grown as above) were assayed for NMT activity (*8*). The total protein content of each lysate was determined by the method of Bradford (*26*). Specific activity is expressed as picomoles of myristoyl-Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg formed per minute per milligram of total protein. The values shown represent those obtained with 10 μg of total lysate protein per assay. Student *t* test (*27*) revealed that the differences between NMT activity in strains with and without additional *NMT1* DNA were significant ($P < 0.05$).

Strain	Copies of *NMT1* per cell	NMT specific activity
YM2061	1	24.1 ± 10.0
YM2061:YEp24	1	23.9 ± 11.9
YM2061:pBB110	8 ± 1	116.6 ± 37.6
YM2061:pBB113	7 ± 1	153.5 ± 11.1

group found on purified NMT was not myristate. Primer extension analysis of total cellular RNA indicated the major transcription start site for this gene occurs 70 to 71 nucleotides upstream of the initiator AUG codon (Fig 1B). Potential TATA transcriptional regulatory elements are located 26 and 67 nucleotides upstream from the deduced mRNA cap site (*11*).

The FASTN and FASTP algorithms did not reveal any significant (*12*) similarities between the nucleotide sequence of the cloned DNA or the primary amino acid sequence of its translation product and any entry in the National Biomedical Research Foundation (NBRF) GenBank (release 55.0) or the NBRF protein sequence data base (release 16.0), respectively. Since NMT binds fatty acyl CoAs, it could have topological similarities with other fatty acid binding proteins that cannot be appreciated by comparison of their primary sequences. Secondary structure predictions of the putative NMT based on the algorithm of Chou and Fasman (*13*) did not resemble those observed in rat intestinal fatty acid binding protein or porcine heart L-3-hydroxyacyl–coenzyme A dehydrogenase. [X-ray crystallographic analyses have recently been used to define the structures of these fatty acid and fatty acyl CoA binding proteins (*14*).] Comparison matrix analyses (*15*) failed to reveal any discernable evidence of internal repeats within the primary structure of the putative NMT. Two predicted sites for N-linked glycosylation are present in this protein sequence (Asn^{114}-Tyr-Thr and Asn^{338}-Asn-Thr), although no information is currently available about the precise compartmentalization of NMT within yeast or whether the enzyme is subjected to this modification.

To directly establish that the product of the cloned gene was NMT, two recombinant DNAs carrying the putative *NMT1* gene were constructed from the yeast episomal plasmid YEp24, which maintains itself at multiple copies per cell (*16*). Cloning of the entire structural gene plus 115 nucleotides of the 5′ nontranscribed region into the tetracycline resistance (*Tc*^{r}) gene of YEp24 created plasmid pBB110. Since it was not known if this construct contained enough promoter sequence for efficient transcription to occur, plasmid pBB113 was generated with an additional 1.15 kb of DNA from the 5′ nontranscribed domain. Strain YM2061 (*7*) was transformed to uracil prototrophy with each of these two constructs and with YEp24 DNA alone. Blots of Bam HI and Bam HI + Sal I–digested total cellular DNA prepared from pBB110 and pBB113 transformants indicated that the sequences cloned into YEp24 were present in approximately seven- to eightfold excess relative to the haploid genomic complement (Table 1).

NMT activity from the three transformants and from the haploid host strain YM2061 was determined as follows. Crude lysates were prepared from cells grown to mid–log phase in synthetic media lacking uracil (except for YM2061) and NMT activity assayed in vitro (*8*) (Table 1). Levels of NMT activity in lysates of YM2061 were similar to those measured in lysates prepared from cells bearing the YEp24 plasmid. However, cells carrying plasmids pBB110 and pBB113 consistently showed a five- to sixfold increase in NMT specific activity over those carrying no plasmid (YM2061) or the parental YEp24 plasmid (Table 1). The pBB113 plasmid, which contains 1.25 kb of 5′ nontranscribed DNA, gave levels of NMT activity that were similar to, or at most 50% higher, than those observed with the pBB110 plasmid that has only 115 nucleotides of promoter sequences (Table 1). The data support the conclusion that the pBB110 plasmid carries the NMT structural gene and that the 115 nucleotides immediately upstream of the transcription initiation site are sufficient to allow efficient gene expression.

Overexpression of NMT in *S. cerevisiae* was not associated with any obvious phenotypic changes. Growth rates and gross cellular morphology were identical between wild-type haploid strains and those that contain five- to sixfold increases in NMT specific activity. Previous studies have shown that distinct subsets of cellular acyl proteins can be identified in yeast by labeling with either [^{3}H]myristate or [^{3}H]palmitate (*8*). To determine if NMT overexpression has any effect on the incorporation of myristate or palmitate into specific acylproteins, strain YM2061 and its YEp24- and pBB113-transformed derivatives were labeled (*8*) with [^{3}H]myristate, [^{3}H]palmitate, or [^{35}S]methionine, and total cellular proteins were subjected to denaturing SDS–polyacrylamide gel electrophoresis and autoradiography. NMT overexpression had no qualitative effects on the distribution of these exogenous ^{3}H-labeled fatty acids in yeast acylproteins.

The ease with which cloned yeast genes can be used to establish null phenotypes allowed us to examine the consequences of eliminating NMT activity in vivo. High-stringency DNA hybridizations indicated that *NMT1* is a single copy gene in *S. cerevisiae* (Fig 2). Single step gene disruption at the *NMT1* locus was performed with the method of Rothstein (*17*). A 1.25-kb Bam HI–Xho I restriction fragment containing the *HIS3* gene encoding imidazoleglycerolphosphate dehydratase was cloned into the unique Eco RV site contained within the *NMT1* coding domain (Fig. 1B), thereby disrupting the gene. Bam HI + Stu I double digests of the resulting construct liberated the disrupted *NMT1* gene from the recombinant plasmid as a linear piece of DNA. This linear DNA has free ends that are suitable for homologous recombination at the *NMT1* locus and was used to trans-

form the diploid strain YB100 (*7*) to histidine prototrophy (Fig 2). Six individual His^+ transformants were screened for a disruption event by probing blots of total cellular DNA with the cloned 2.1-kb Bam HI–Hind III genomic restriction fragment encompassing the entire *NMT1* structural gene. All six transformants gave the expected size restriction fragments predicted to result from a single copy integration event of the *HIS3* gene into one of the two *NMT1*

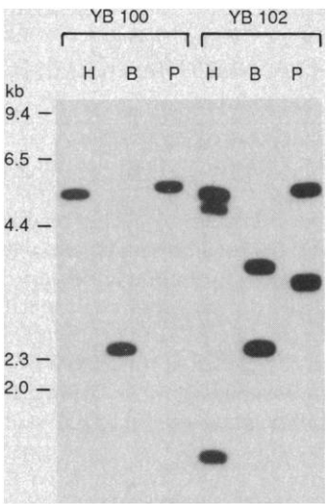

Fig. 2. Disruption of the *NMT1* gene causes recessive lethality. pBB105 is a pUC13 recombinant plasmid that has the *NMT1* coding region plus 115 nucleotides of 5′ and 556 nucleotides of 3′ flanking DNA cloned as a Bam HI–Pst I fragment (derived from the 5.4-kb Hind III genomic clone shown in Fig. 1A). The ends of a Bam HI–Xho I restriction fragment containing the *HIS3* gene plus ~250 nucleotides of 5′ upstream DNA (*30*) were blunt ended with the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs) and all four deoxynucleoside triphosphates. Subsequent cloning of this fragment into the Eco RV site in the *NMT1* gene (Fig. 1B) of pBB105 created plasmid pBB109. Bam HI + Stu I cut pBB109 was used to transform the diploid strain YB100 (*7, 19*) to His^+, and transformants YB101 and YB102 carrying the mutant *nmt::HIS3* allele were isolated. Total genomic DNA was prepared from strains YB100 (*NMT1/NMT1*) and YB102 (*NMT1/nmt::HIS3*) (*25*), digested with the indicated enzymes, subjected to electrophoresis through a 0.8% agarose gel, blotted to Gene Screen Plus (Du Pont), and probed with the *NMT1* gene. Twice as much YB102 DNA was loaded per lane as YB100 so individual band intensities would be similar. The growth kinetics of strains YB100 and YB102 were identical. H, Hind III; B, Bam HI; and P, Pst I.

Distribution of the numbers of viable spores within tetrads of YB101 and YB102.

Strain	Number of viable spores within tetrads				
	4	3	2	1	0
YB101	3	2	87	21	3
YB102	1	1	96	8	1

alleles of YB100 (Fig. 2) (*18*). There was no indication of any integration event other than that at the *NMT1* locus in any of the six independent strains analyzed. Two of these strains (YB101 and YB102) were saved for further analysis.

To determine the biological consequences of the *NMT1* disruption, strains YB101 and YB102 were sporulated on nitrogen-deficient media and over 100 four-spore tetrads from each strain were dissected on yeast-peptone dextrose plates (*19*). After 3 to 4 days' growth at 30°C, nearly 90% of the tetrads segregated with a viable:nonviable ratio of 2:2, revealing that the Nmt^- phenotype is lethal (table in legend to Fig. 2). Eight percent of the spores failed to germinate in control tetrad dissections of sporulated YB100, accounting for the tetrads that segregated 1:3. The rare (3%) 4:0 and 3:1 segregants may have arisen from mitotic crossing over or gene conversion events (*20*). All viable spores were phenotypically His^-, showing tight linkage between the *HIS3* gene and the recessive lethal mutation. Spores lacking functional NMT enzyme were able to germinate but ceased to divide after one to four cell divisions. This "micro" colony formation is not an uncommon occurrence among recessive lethal mutations (*21*) and may represent spore inheritance of maternal NMT and/or critical myristoyl proteins that are diluted or degraded through subsequent cell divisions until they fall below essential intracellular concentrations. To assess whether growth arrest always occurred at the same stage of the cell cycle, colonies of dead cells were physically separated by micromanipulation (*19*) and examined under a microscope. Of the 135 cells scored, 33% had no visible bud and the rest had bud sizes ranging from barely detectable to the same as that of the mother cell.

These experiments suggest that NMT is necessary for vegetative growth of haploid yeast cells. NH_2-terminal myristoylation must provide a critical function for those proteins subjected to this covalent modification, since an *NMT1* disruption mutation causes recessive lethality. Although none of the yeast cellular myristoyl proteins have been definitively identified, candidates include the ARF and GPA1 (SCG1) guanine nucleotide binding proteins, which have myristoylation consensus sequences (*6, 22*).

Functional replacement of a mutant gene product by a structurally distinct protein may partially contribute to the observation that 70% of the yeast genomic sequences are nonessential for cell growth and division (*23*). Yeast possess an acyltransferase activity responsible for attachment of palmitate in ester linkage to cysteine, serine, or threonine residues (*2, 8*). The results of the *NMT1* disruption experiment reported here indicate that palmitoyl transferase cannot substitute for NMT activity in vivo, highlighting the uniqueness of each acyltransferase and its form of protein acylation.

Preliminary mapping studies indicate that the *NMT1* locus is located on the right arm of yeast chromosome XII (*24*). A more detailed description of the *NMT1* map location will be required for subsequent genetic analysis. The availability of a cloned yeast *NMT1* DNA should permit overexpression, isolation, and crystallization of NMT, a necessary step for fully defining its structure-activity relationships. Experiments designed to identify myristoyl proteins crucial for cell viability or to define the cellular machinery that interacts with the myristoyl moiety of these proteins become possible with the acquisition of the *NMT1* gene.

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18. Bam HI fails to cut within the inserted *HIS3* sequences and thus produced two reactive bands: a fragment from the wild-type *NMT1* allele and another 3.75-kb fragment from the disrupted allele. The 5.0-kb and 1.4-kb Hind III bands appear in addition to the 5.4-kb wild-type Hind III band, since two new Hind III sites were introduced by the *HIS3* coding region. A 3.5-kb Pst I band is generated by the addition of a Pst I site near the 3′ end of the inserted *HIS3* DNA: integration of the *HIS3* sequences should increase the length of the wild-type 5.7-kb Pst I fragment to 7 kb, but the new Pst I site bisects this and the intensity of the 3.5-kb band is increased, as expected, because of the doublet.
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Isolation of a Novel Receptor cDNA Establishes the Existence of Two PDGF Receptor Genes

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A genomic sequence and cloned complementary DNA has been identified for a novel receptor-like gene of the PDGF receptor/CSF1 receptor subfamily (platelet-derived growth factor receptor/colony-stimulating factor type 1 receptor). The gene recognized a 6.4-kilobase transcript that was coexpressed in normal human tissues with the 5.3-kilobase PDGF receptor messenger RNA. Introduction of complementary DNA of the novel gene into COS-1 cells led to expression of proteins that were specifically detected with antiserum directed against a predicted peptide. When the new gene was transfected into COS-1 cells, a characteristic pattern of binding of the PDGF isoforms was observed, which was different from the pattern observed with the known PDGF receptor. Tyrosine phosphorylation of the receptor in response to the PDGF isoforms was also different from the known receptor. The new PDGF receptor gene was localized to chromosome 4q11–4q12. The existence of genes encoding two PDGF receptors that interact in a distinct manner with three different PDGF isoforms likely confers considerable regulatory flexibility in the functional responses to PDGF.

SEVERAL FAMILIES OF MEMBRANE-spanning growth factor receptors with tyrosine kinase activity have been identified (*1*). One such family includes the structurally related growth factor receptors for platelet-derived growth factor (PDGF) (*2*) and colony-stimulating factor (CSF1) (*3*). The latter was initially identified as the normal cellular homolog of the v-*fms* oncogene (*4*). A third member of this family, c-*kit* (*5*), for which the ligand has yet to be identified, was also first detected as a viral oncogene (*6*). In the present studies, we describe the identification of a genomic sequence and the isolation of the cDNA of a novel gene, whose predicted product has a structure similar to other growth factor receptors of this subfamily.

Reduced stringency hybridization of human genomic DNA with DNA probes from the tyrosine kinase domain of v-*fms* or of the mouse PDGF receptor (PDGFR) led to detection of several bands in addition to those of either c-*fms* or PDGFR genes. A 12-kbp Eco RI band generated the strongest signal with either probe, while smaller size bands corresponded in size to restriction fragments reported for human c-*kit* (*6*). Thus, we cloned the 12-kbp Eco RI fragment, which we designated λT11. Regions homologous to v-*fms*/PDGFR tyrosine kinase domains within λT11 were localized by hybridization and subjected to nucleotide sequence analysis. Their discrete open reading frames were most closely related to but distinct from human c-*fms*, c-*kit*, or the PDGFR. To characterize the coding region of this gene, overlapping cDNA clones were isolated from normal human fibroblast and brain cDNA libraries and subjected to nucleotide sequence analysis. For structural and functional comparison, we also cloned and sequenced cDNAs encompassing the entire coding sequence of the human PDGF receptor (*7*).

For the novel gene, a single open reading frame of 1089 amino acids was predicted from the nucleotide sequence, which has been submitted to GenBank (*8*). According to the putative cleavage site for the hydrophobic signal peptide (*9*), the NH_2-terminus of the mature product was predicted to be glutamine at amino acid 24 followed by 1065 amino acids. This polypeptide sequence with a calculated molecular mass of around 120 kD contained all of the characteristics of a membrane-spanning tyrosine kinase receptor. A hydrophobic segment consisting of 24 amino acids (residues 525

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