THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 44, Issue of November 3, pp. 26252–26256, 1995
Printed in U.S.A.

Cloning of a cDNA Encoding Chitotriosidase, a Human Chitinase Produced by Macrophages*

(Received for publication, June 29, 1995, and in revised form, August 25, 1995)

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We have recently observed that chitotriosidase, a chitinolytic enzyme, is secreted by activated human macrophages and is markedly elevated in plasma of Gaucher disease patients (Hollak, C. E. M., van Weely, S., van Oers, M. H. J., and Aerts, J. M. F. G. (1994) *J. Clin. Invest.* 93, 1288–1292). Here, we report on the cloning of the corresponding cDNA. The nucleotide sequence of the cloned cDNA predicts a protein with amino acid sequences identical to those established for purified chitotriosidase. Secretion of active chitotriosidase was obtained after transient transfection of COS-1 cells with the cloned cDNA, confirming its identity as chitotriosidase cDNA. Chitotriosidase contains several regions with high homology to those present in chitinases from different species belonging to family 18 of glycosyl hydrolases. Northern blot analysis shows that expression of chitotriosidase mRNA occurs only at a late stage of differentiation of monocytes to activated macrophages in culture. Our results show that, in contrast to previous beliefs, human macrophages can synthesize a functional chitinase, a highly conserved enzyme with a strongly regulated expression. This enzyme may play a role in the degradation of chitin-containing pathogens and can be used as a marker for specific disease states.

Chitinases are known to occur in a variety of species, including bacteria, fungi, nematodes, plants, insects, and fish (1). It has often been suggested that man and other mammals do not possess an analogous chitinase (2, 3). We have recently reported on the existence of a chitinolytic enzyme in man, designated as chitotriosidase, which is secreted in large quantities by activated macrophages (4). Both N-terminal and internal amino acid sequences of purified chitotriosidase suggest a homology with proteins of the chitinase family, including enzymatically inactive members (5).

The enzyme chitotriosidase is also of interest for clinical reasons. Chitotriosidase activity is several hundred-fold elevated in plasma of patients suffering from Gaucher disease, a disorder characterized by the presence of large amounts of activated, lipid-laden macrophages in spleen, liver, and other tissues (4). More modest elevations are found in plasma of patients with sarcoidosis and leishmaniasis (4). The enzyme can therefore serve as a valuable diagnostic marker particularly for Gaucher disease and may be useful in monitoring the efficacy of therapy. Furthermore, evidence has been obtained that a genetic deficiency of chitotriosidase occurs with high frequency in man; the nature and consequence of this defect is not yet known (4).

To obtain more insight into the structural features and regulation of the expression of human chitotriosidase, we have cloned the corresponding full-length cDNA. This enabled us to compare the derived primary structure of chitotriosidase with that of other proteins of the chitinase family and to monitor the expression of chitotriosidase mRNA in a model system consisting of cultured human peripheral blood monocytes differentiating into activated macrophages. The results are described in this paper.

MATERIALS AND METHODS

In Vitro Differentiation of Peripheral Blood Monocytes to Macrophages—Human peripheral blood monocytes were isolated and cultured as described (4). Spontaneous differentiation of monocytes into activated macrophages occurs upon prolonged cell culture (4).

Polymerase Chain Reaction Amplification of a Chitotriosidase cDNA Probe—Total RNA was isolated from cultured macrophages that secreted large amounts of chitotriosidase activity as described (6, 7). First strand cDNA synthesis was performed on 15 μg of total RNA using SuperScript™ RNase H$^-$ reverse transcriptase (Life Technologies, Inc.) and oligo(dT). After alkaline hydrolysis, the cDNA was precipitated with ethanol and used as template for polymerase chain reaction (PCR)[1] amplification with degenerate chitotriosidase primers. Primers, manufactured by Pharmacia, were as follows: 5′-TGYTAYTTYAC-NAAYTGGGC-3′ (N-terminal derived sense primer) and 5′-CCART-CIARRTYIAVICCRTCRAA-3′ (an antisense primer designed on the basis of homology within the chitinase family). PCR was performed using standard conditions except for annealing at 48 °C. After 25 cycles a DNA fragment of the expected size was obtained, and subsequently the fragment was purified, treated with T4 DNA polymerase (Pharmacia, Uppsala, Sweden), and cloned into the *Hin*cII site of plasmid pUC19. DNA sequence analysis was performed using the dideoxy-nucleotide chain termination method with T7 polymerase (Pharmacia) and the M13 forward and reverse primers (New England Biolabs, Beverly, MA) as described (7, 8). The cloned PCR fragment of chitotriosidase was used as probe in the cDNA library screening.

Isolation of a Full-length Chitotriosidase cDNA from a Macrophage cDNA Library—Double-stranded macrophage cDNA was prepared from total macrophage RNA using the SuperScript Choice System cDNA synthesis kit (Life Technologies, Inc.) according to the manufacturer's protocol. Double-stranded cDNA was ligated to an excess of non-palindromic *Bst*XI linkers and subsequently size fractionated on a low melting-type agarose gel, cDNA exceeding 500 base pairs was purified and ligated into the *Bst*XI sites of the vector pcDNA1 (Invitrogen). The ligation mixture was electroporated into *Escherichia coli* strain MC1063/p3 to obtain a macrophage cDNA library. The cDNA library was screened by colony hybridization using the partial chitotriosidase cDNA probe, which had been radiolabeled with ^{32}P by the random

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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) U29615.

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[1] The abbreviation used is: PCR, polymerase chain reaction.

priming method (7). Hybridization to the radiolabeled probe was carried out for 4 h in 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, and 7% SDS at 65 °C. Subsequently, the filters were washed twice in 1 × SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS and subjected to autoradiography. The positive clones were sequenced as described above.

Computer Analysis—Nucleotide and amino acid homology searches were carried out using the program BLAST (Basic Local Alignment Search Tool (9)). Sequence analysis of cDNA clones was performed using Mac Vector 4.1.1 and the Genetics Computing Group package (version 7.0). Sequence comparisons and multiple sequence alignments of members of the chitinase family of proteins were performed using the multiple sequence alignment program PILEUP and BESTFIT.

Activity Measurement of Recombinant Chitotriosidase—Transient transfection of COS-1 cells by the DEAE-Dextran method was performed essentially as described in Ref. 10. The activity of chitotriosidase was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose (Sigma) exactly as described before (4). For the experiments, medium from transfected COS cells was collected, and cells were harvested. Transfected COS cells were harvested by trypsinization and extracted by sonication in 0.25% (v/v) Triton X-100, 50 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged, and the supernatant was collected.

Northern Blot Analysis—Total macrophage RNA was isolated using RNAzol B (Biosolve, Barneveld, The Netherlands) according to the instructions of the manufacturer. Samples (20 μg) were electrophoresed in 10 mM Hepes, 6% formaldehyde-agarose gels, transferred to Hybond N nylon membranes (Amersham, Buckinghamshire, United Kingdom) by the capillary method, and immobilized by UV cross-linking. The following ^{32}P-radiolabeled probes were used: total chitotriosidase cDNA, a PCR fragment of type 5, tartrate-resistant acid phosphatase cDNA, and mouse glyceraldehyde-3-phosphate-dehydrogenase cDNA. Hybridization conditions were as described above for the library screening. The signals on the membranes were quantitated using a PhosphorImager (Molecular Dynamics).

RESULTS

Strategy for the Molecular Cloning of the Chitotriosidase cDNA—First, a probe to identify chitotriosidase cDNA was generated. The previously established N-terminal amino acid sequence of chitotriosidase was used to design a degenerate sense oligonucleotide. A degenerate antisense oligonucleotide was designed on the basis of a highly conserved domain in the chitinase family. These oligonucleotides were used to amplify a DNA fragment by reverse transcriptase-PCR using total RNA isolated from cultured macrophages as template. The resulting DNA fragment was of the expected size (on the basis of homology with members of the chitinase family) and was cloned into the plasmid vector pUC19. Determination of its sequence revealed that the fragment was in complete accordance with the known N-terminal amino acid sequence of purified human chitotriosidase (5), allowing its use as a probe to identify a full-length chitotriosidase cDNA.

A cDNA library was prepared using total RNA from cultured macrophages. About 0.1% of the colonies were positive upon hybridization with the probe. One of them had an insert of 1.7 kilobase pairs, which is large enough to contain the complete coding information for chitotriosidase. The nucleotide sequence of this cloned cDNA revealed an open reading frame of 1398 base pairs long, starting with an ATG codon at position 13 and ending with a TGA codon at position 1410 (Fig. 1). The open reading frame encodes a protein with a characteristic N-terminal 21 amino acids endoplasmic reticulum signal peptide immediately followed by the N-terminal sequence established for the chitotriosidase protein (5). The cDNA sequence does not indicate the presence of potential *N*-linked glycosylation sites, which is consistent with the absence of *N*-linked glycans in isolated chitotriosidase (5). The predicted protein, after removal of the signal sequence, has a length of 445 amino acids and a calculated molecular mass of 49 kDa, thus corresponding to the larger of the two major isoforms of chitotriosidase isolated previously from Gaucher spleen.

Homology with Other Proteins—A search of the EMBL and GenBank data bases revealed significant homology with a group of chitinases and related proteins from different species (see Fig. 1). The strongest homology was observed with a recently published cDNA sequence, for a chitinase-related human cartilage glycoprotein (11), and with a cDNA sequence coding for a secretory protein from activated mouse macrophages (GenBank accession no. M94584). These two proteins have no chitinolytic activity, and their function is not known (Ref. 11 and GenBank accession no. M94584). The human chitotriosidase cDNA sequence indicates the presence of a region that is highly homologous to the presumed catalytic center in chitinases (Fig. 2; see Ref. 15).

A comparison with amino acid sequences of members of the chitinase family, also including the enzymatically inactive proteins, reveals additional homologous regions. In Fig. 1, the amino acids in chitotriosidase that are identical to those in at least six out of nine members of the chitinase protein family are indicated by *bold characters*.

The predicted C-terminal part of human chitotriosidase shows only homology with two members of this family, *Manduca sexta* (13) and *Brugia malayi* chitinases (14). The homologous C-terminal stretches in these chitinases are characterized by the abundant presence of serine residues that might be modified by glycosylation. In the case of the *B. malayi* chitinase, *O*-linked glycosylation has indeed been demonstrated (14).

Functional Expression of Chitotriosidase in COS Cells—Expression of chitotriosidase upon transient transfection of COS-1 cells with the full-length chitotriosidase cDNA was monitored by detection of enzyme activity. At day 7 post-transfection, chitotriosidase activity was present in the medium of the transfected COS cells. No activity was detectable in mock-transfected COS cells or cells transfected with the same cDNA inserted in the antisense orientation. This enzyme activity produced by transfected COS cells could be inactivated in an identical manner as splenic chitotriosidase, with a rabbit polyclonal antiserum raised against purified human tissue chitotriosidase (data not shown).

Expression of Chitotriosidase mRNA in Monocyte-derived Macrophages—Northern blot analysis with the chitotriosidase and tartrate-resistant acid phosphatase cDNA probes was used to investigate mRNA expression during differentiation of cultured blood monocytes into macrophages (see Fig. 3). Tartrate-resistant acid phosphatase mRNA, a differentiation marker for macrophages, was already detectable after 2 days of culture and gradually increased in time, reaching a maximum level at 14 days of culture (*panel A*). During the first 4 days of culture, chitotriosidase mRNA was not detectable. Only at day 7, mRNA for chitotriosidase became detectable and increased markedly in time. Even at the end of the experiment, day 22 of culture, a steady state level of chitotriosidase mRNA had not been reached (*panel B*). This time dependence is similar to that observed for the production of chitotriosidase activity in the cell culture model (see Ref. 4).

DISCUSSION

Several lines of evidence show that the isolated cDNA clone encodes chitotriosidase. First, N-terminal and internal peptide sequences of purified tissue chitotriosidase are present in the protein sequence derived from the cloned cDNA. Second, transfection of COS-1 cells with the cDNA results in the synthesis and secretion of an enzymatically active chitinase that, like splenic chitotriosidase, is inactivated by antiserum raised against the tissue enzyme. Furthermore, we observed that the relative rate of hydrolysis of the artificial substrates 4-methylumbelliferyl-chitotrioside and 4-methylumbelliferyl-chitobio-

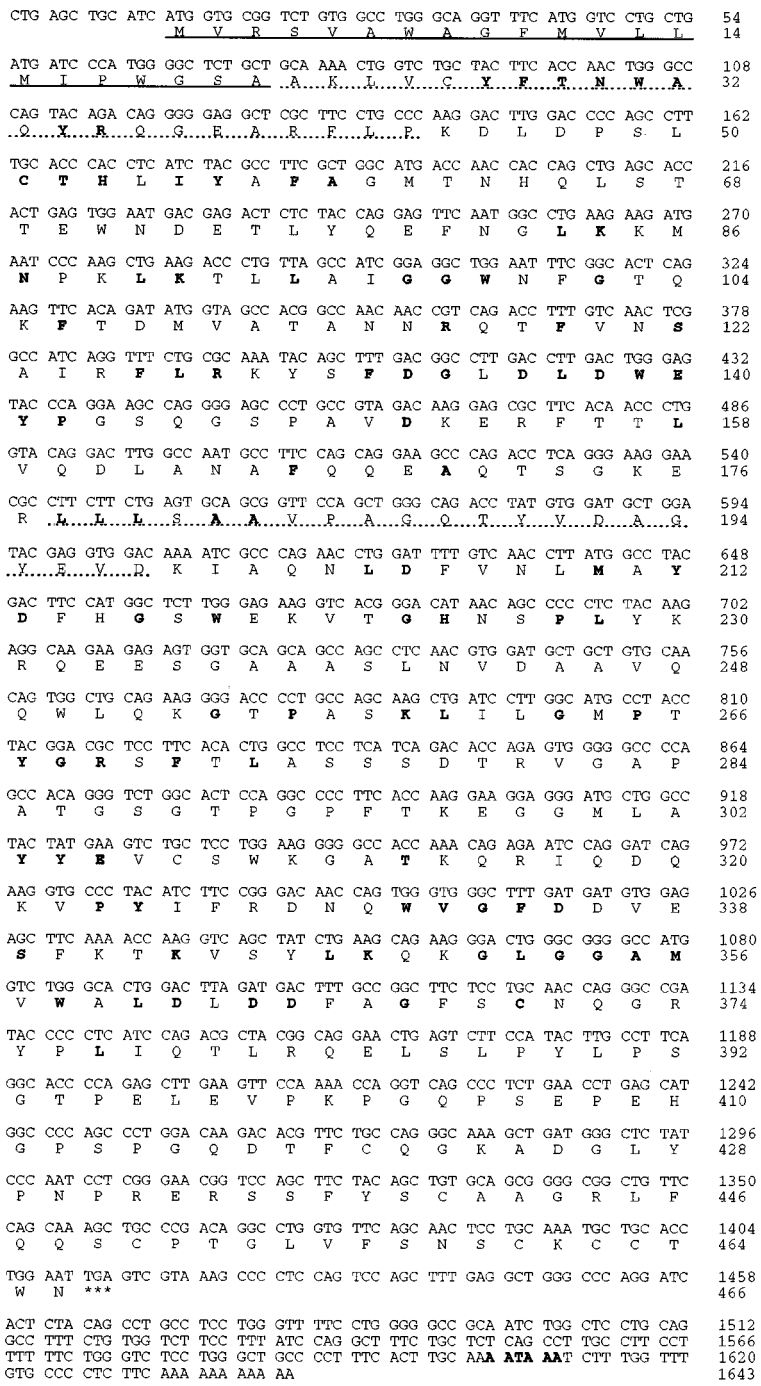

FIG. 1. **Chitotriosidase cDNA sequence and deduced amino acid sequence.** The hydrophobic leader peptide (amino acids 1–21) is *underlined*. Amino acids in chitotriosidase that are identical to those in at least six out of nine members of the chitinase family are depicted in *bold characters*. The nine members of the chitinase family used are listed in the legend of Fig. 2, with the exception of *Autographa californica* chitinase and *Nicotiana tabacum* chitinase. The stretches of amino acids that are also identified in splenic enzyme are *underlined* with *dots* (5). The putative polyadenylation signal is indicated in *bold*. The chitotriosidase cDNA sequence has been deposited in the GenBank data base.

side was identical for tissue chitotriosidase and enzyme produced by COS cells (data not shown). Finally, the absence of *N*-linked glycans in chitotriosidase is predicted by the cDNA sequence, which is consistent with the results of our previous investigations on purified chitotriosidase (5).

The results of our study imply that there is a gene in man encoding a chitinolytic enzyme homologous to chitinases from various other species, in particular with respect to the region that is assumed to be an essential part of the catalytic site (15). Strong homologies between the human enzyme and other chitinases are also noted for various other regions that might be crucial to generate the characteristic conformation of this type of enzyme.

The extensive homology between human and other chitinases is interesting and may have important clinical implications. For instance, the use of parasite chitinase fragments as vaccine targets has been proposed (1, 16). It may be advisable to use only non-homologous regions of parasite chitinases for vaccination to prevent a potential immunological response to the endogenous human counterpart.

On the basis of its sequence homology with other chitinases, the human chitotriosidase should be considered to belong to family 18 of glycosyl hydrolases (17). It has recently been proposed that the catalytic core structure of family 18 of glycosyl hydrolases is an 8-stranded α/β (TIM) barrel (18). Although some plant endochitinases and lysozyme belong to a family of glycosyl hydrolases distinct from family 18, we have observed some similarities between human chitotriosidase and lysozyme (19). For instance, chitotriosidase, like lysozyme, is relatively heat stable and resistant to proteolytic degradation.

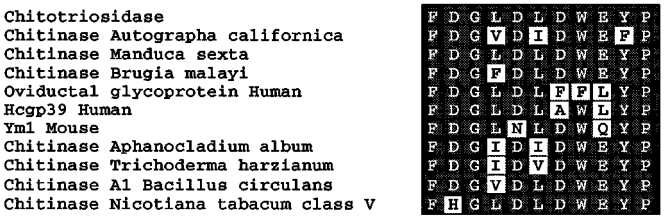

FIG. 2. **Alignment of putative active site regions in members of the chitinase protein family.** The proteins are as follows: chitotriosidase and a chitinase of the virus *A. californica* (GenBank L22858), a chitinase of the tobacco hornworm *M. sexta* (GenBank U02270), an endochitinase of the nematode *B. malayi* (GenBank M73689), a human oviduct-specific glycoprotein (GenBank U09550), human cartilage gp-39 (a human glycoprotein produced by chondrocytes and synovial cells (GenBank M80927)), YM-1 (a secretory protein of activated mouse macrophages (Pir S27879)), a chitinase of the fungus *Aphanocladium album* (SwissProt P32470), a chitinase of the filamentous fungus *Trichoderma harzianum* (GenBank L14614), chitinase A1 from the prokaryote *Bacillus circulans* (SwissProt P20533), and a class V chitinase from the plant *N. tabacum* (GenBank X77110). Residues identical to chitotriosidase are indicated by the *reverse type*. The human oviductal glycoprotein, human cartilage gp-39, and YM-1 are supposedly not chitinolytic.

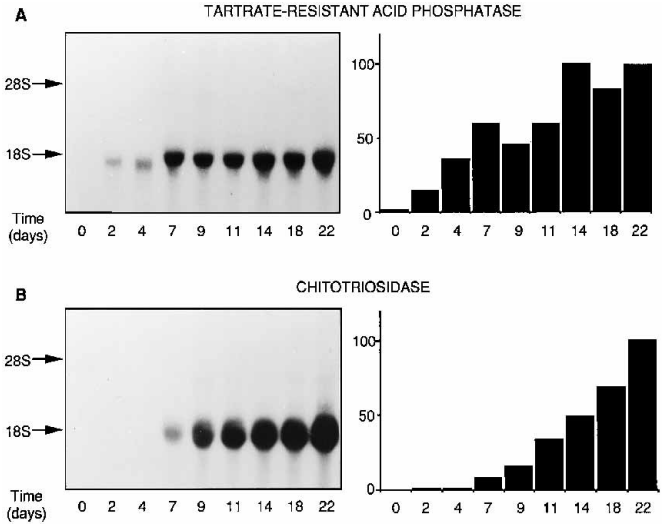

FIG. 3. **Time-related changes in expression of mRNA for chitotriosidase and tartrate-resistant acid phosphatase in monocyte-derived macrophages as monitored by Northern analysis.** Human peripheral blood monocytes were isolated and cultured as described under "Materials and Methods." Total RNA was extracted at the time points indicated and analyzed as described under "Materials and Methods." The positions of the 28 S and the 18 S rRNA bands are indicated with *arrows*. The same blot was used for hybridization with the different probes. The radioactive signals for acid phosphatase (*upper graph*) and chitotriosidase mRNA (*lower graph*) were related to that of glyceraldehyde-3-phosphate dehydrogenase mRNA in the same lane (*right panels*). The level of glyceraldehyde-3-phosphate dehydrogenase mRNA over total RNA was constant in time. The relative intensities of the signals in *panels A* and *B* cannot be compared because of differences in the specific activities of the probes.

Chitotriosidase, like lysozyme, is inhibited in activity by high concentrations of the substrate 4-methylumbelliferyl-chitotrioside and shows a very broad pH optimum. Chitotriosidase and lysozyme are both present in lysosomes and granules of neutrophilic granulocytes. Macrophages constitutively secrete both chitotriosidase and lysozyme and partly accumulate the enzymes in their lysosomes.[2] The analogies between chitotriosidase and other members of family 18 of glycosyl hydrolases, and some of its similarities with the well characterized lysozyme, should be helpful in future investigations on structure-function relationships in chitotriosidase.

Chitotriosidase, unlike most lysosomal hydrolases, is not a house-keeping enzyme. The macrophages are capable of producing very large amounts of the enzyme under specific circumstances. One such condition is the prolonged culture of macrophages derived from peripheral blood monocytes. On the basis of our findings, it may be assumed that chitotriosidase mRNA comprises approximately 0.1% of the total mRNA in macrophages cultured for 3 weeks. Metabolic labeling studies suggest that about 1% of the secretory proteins of these cells is chitotriosidase. Another condition is found in Gaucher disease, in which an inherited deficiency in glucocerebrosidase activity causes lysosomal storage of glucosylceramide in macrophages. This is accompanied by greatly enhanced levels of chitotriosidase in plasma (4).

The physiological role of the human chitinase still has to be established. On the basis of its known properties, it seems likely to fulfill a role in the degradation of chitin-containing pathogens. The existence of a rather frequent, inherited deficiency in chitotriosidase activity in man and the occurrence of abnormal high enzyme levels in plasma of Gaucher disease patients should help to elucidate the physiological role of chitotriosidase (4). In this connection, it is interesting to note that plants are known to produce high amounts of chitinases under conditions of stress (20, 21). The anti-fungal action of these proteins in plants is very well documented (22–24). More recently, an additional role in morphogenetic processes in plants has been postulated (12, 21). The exact function of chitinases in morphogenesis is not clear, since plants are believed to contain no endogenous chitin or analogous structures (21). It is conceivable that chitotriosidase in man may also have a dual function. Alternatively, homologous proteins may be involved in morphogenetic events in man. The homologous human cartilage gp-39 is a 39-kDa glycoprotein that is produced by articular chondrocytes and synovial cells under specific conditions, as in rheumatoid arthritis. A role for this protein, which lacks chitinolytic activity, has been proposed in tissue remodeling (11).

The demonstration of the existence of a human chitinase warrants further investigation. The role of chitotriosidase in defense against specific pathogens has to be carefully examined as well as the possibility of another physiological function. Furthermore, the finding that chitotriosidase production is associated with the presence of specifically stressed macrophages may be exploited to monitor progression and correction of a number of pathological conditions in man, such as Gaucher disease, in which these types of cells are involved.

Acknowledgments—We thank Inge Pool and Wilma Donker for skillful assistance. Dr. J. M. Tager, Dr. H. F. Tabak, and Dr. S. van Weely are acknowledged for comments and suggestions during the preparation of the manuscript.

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J. Biol. Chem. 1995, 270:26252-26256.
doi: 10.1074/jbc.270.44.26252