The gene encoding DRAP (BACE2), a glycosylated transmembrane protein of the aspartic protease family, maps to the Down critical region

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Abstract We applied cDNA selection methods to a genomic clone (YAC 761B5) from chromosome 21 located in the so-called 'Down critical region' in 21q22.3. Starting from human fetal heart and brain mRNAs we obtained and sequenced several cDNA clones. One of these clones (Down region aspartic protease (DRAP), named also BACE2 according to the gene nomenclature) revealed a striking nucleotide and amino acid sequence identity with several motifs present in members of the aspartic protease family. In particular the amino acid sequences comprising the two catalytic sites found in all mammalian aspartic proteases are perfectly conserved. Interestingly, the predicted protein shows a typical membrane spanning region; this is at variance with most other known aspartic proteases, which are soluble molecules. We present preliminary evidence, on the basis of in vitro translation studies and cell transfection, that this gene encodes a glycosylated protein which localizes mainly intracellularly but to some extent also to the plasma membrane. Furthermore DRAP/BACE2 shares a high homology with a newly described β-secretase enzyme (BACE-1) which is a transmembrane aspartic protease. The implications of this finding for Down syndrome are discussed.

Key words: Aspartic protease; Enzyme; cDNA; Chromosome 21; Down syndrome

1. Introduction

The endopeptidases dependent on aspartic residues for their catalytic activity (EC 3.4.23) belong to three main families which are those of the pepsin (A1), retropepsin (A2) and that of the pararetroviruses such as the cauliflower mosaic virus (A3) [1]. The A1 family comprises several members, among which the widely known cathepsin D, cathepsin E, chymosin, gastricsin, pepsin A, pepsin F and renin are all related by extensive sequence homology [1–3].

Similarities in three-dimensional (3D) structures [4–7] together with sequence homologies indicate that all aspartic proteases are likely to resemble one another very closely. In particular the refined structures for fungal/vertebrate enzymes are broadly similar with dimensions of ca 65×40×30 Å; these proteases are bilobed molecules with the active-side cleft located between the lobes and with each lobe contributing one of the pair of aspartic acid residues responsible for the catalytic activity [8,9]. Furthermore, the catalytic aspartic acid residues occur within the motif Asp-Ser/Thr-Gly in both N- and C-terminal lobes of the enzyme. This structural organization is most likely derived from gene duplication events [3]. Recently a new member of the family has been described which shows an unprecedented transmembrane spanning domain [10]. This protein cleaves the amyloid precursor protein (APP) at the NH2 terminus of the amyloid β peptide Aβ to release APPsβ, a 100 kDa soluble NH2-terminal fragment, leaving a 12 kDa COOH-terminal fragment (C99) bound to the membrane [11]. The new protein was called BACE-1 for β site APP-cleaving enzyme [10].

We present here the cDNA sequence of a gene sharing several features with the members of the A1 family of aspartic proteases and in particular with BACE-1. Interestingly the gene encoding the new cDNA is located on the long arm of chromosome 21 in the so called 'Down critical region' at 21q22.3 and was therefore dubbed DRAP for Down region aspartic protease. We have also partially characterized the protein product encoded by the cloned cDNA.

2. Materials and methods

2.1. Direct cDNA selection

Poly(A)+ mRNA was prepared from human fetal heart and brain with the oligotex mRNA kit (Promega). 5 μg of mRNA were reverse transcribed into double strand cDNA, which was digested with *Mbo*I and ligated to synthetic oligonucleotide linkers as previously described [11].

The human chromosome 21-derived YAC 761B5 is a non-chimeric clone that was obtained from the CEPH library. YAC DNA was separated from the endogenous yeast chromosomes by preparative pulsed field gel electrophoresis (PFGE) and purified [12]. The enrichment for transcripts deriving from genes mapping to the YAC was achieved essentially as described [11].

2.2. In vitro transcription and translation

Plasmid pSP64Tnew was derived from pSP64T [13] by linearization with *Bgl*II and insertion of a synthetic polylinker carrying the recognition sites for the restriction enzymes *Apa*I, *Xho*I, *Hpa*I and *Not*I. PSP64Tnew DNA was digested to completion with *Not*I, blunted with Klenow enzyme and digested with *Apa*I prior to agarose gel purification. The cDNA clone containing the putative aspartic protease ORF was digested with *Xba*I and the DNA was blunted with Klenow enzyme and digested with *Apa*I thus producing a 1710 bp DNA fragment (containing the whole ORF) which was gel purified and ligated to the pSP64Tnew vector. The plasmid DNA was then linearized by

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*Bam*HI digestion and transcribed in vitro with SP6 RNA polymerase in the presence of m7GpppG for 90 min at 40°C as described [14]. The RNA was extracted once with 1:1 phenol-chloroform, once with chloroform and finally once with dimethylether before being ethanol precipitated with 0.3 M sodium acetate and resuspended in DEPC-treated water in the presence of 1 unit/μl of RNasin. In vitro translation was performed with wheat germ extracts (Promega) in the presence of ^{35}S-labeled methionine according to the supplier's protocol. 1–2 μg of transcribed RNA were used in a 25 μl reaction that was allowed to proceed for 1 h at 25°C.

When indicated, 1 μl of Canine Microsomal Membranes (Promega) was included in the reaction. The samples were then analyzed by SDS–PAGE on a 15% polyacrylamide gel followed by autoradiography. Proteinase K treatment of translation reactions was performed at a final concentration of 100 μg/ml for 30 min on ice in the presence or absence of 0.5% Triton X-100, immediately followed by protease inactivation with 5 mM phenylmethylsulfonyl fluoride. Endo H treatment was performed with the deglycosilation kit from Boehringer Mannheim according to the manufacturer's instructions; briefly, 5 μl of the translation mixture were incubated at 95°C for 3 min in the presence of one volume of buffer 2, then 10 μl of reaction buffer and 10 μl of Endo H were added to the sample and incubated at 37°C for different times; control reactions contained no Endo H enzyme. Translocation of polypeptides to the luminal side of the microsomes was further confirmed by prevention of *N*-linked glycosylation through competitive inhibition following the inclusion in the in vitro translation reaction of the glycosylation acceptor tripeptide (acetyl)-Asn-Tyr-Thr-(amide) [15]. As an independent assay for *N*-glycosylation, the β-glucosidase inhibitor *N*-methyl deoxynojirimycin (Hettkamp et al., 1984) was included in the in vitro translation reaction at a final concentration of 3 mM. To analyze the translocated proteins under alkaline conditions, a 10 μl aliquot of the proteins translated in the presence of microsomal membranes was incubated on ice with 490 μl of 100 mM Na_2CO_3 (pH 11.5) for 30 min, pelleted by centrifugation at 100 000 × *g* for 30 min and rinsed with ice-cold PBS before SDS–PAGE analysis.

The supernatant was also collected and its content precipitated with 10% TCA before electrophoresis.

2.3. Sequence analysis of DRAP-1/BACE2

Protein sequence alignments were initially performed with the program Clustal W [16] (http://www.infobiogen.fr/docs/ClustalW/clustalw.html), and then manually modified with the sequence editor Seaview [17] (ftp://pbil.univ-lyon1.fr/pub/mol_phylogeny/seaview), taking into consideration the alignment of the DRAP-1 sequence to those of aspartic proteases of known structure obtained from the SWISS-MODEL program. Secondary structure predictions were performed using the programs Predator [18] (http://www.embl-heidelberg.de/cgi/predator_serv.pl) and PHDsec [19] (http://www.embl-heidelberg.de/predictprotein/predict protein.html). In order to evaluate whether the DRAP-1 amino acid sequence could be compatible with the conserved fold of the aspartic protease protein class, its fold was predicted using three different recognition algorithms: 3D-PSSM [20] (http://bonsai.lif.icnet.uk/foldfitnew/index.html), Gon+predss [21] (http://fold.doe-mbi.ucla.edu/Home) and TOPITS [22] (http://www.embl-heidelberg.de/predict protein/predictprotein.html).

3. Results

3.1. Identification of DRAP/BACE2

Two YAC clones from chromosome 21 (761B5 and 773A5 clones from the CEPH library) which are adjacent in 21q22.3 and span approximately 1800 kb of genomic DNA were selected for gene isolation. Human mRNAs from endocardium, myocardium and total brain were used for cDNA selection procedures and several clones were obtained, sequenced and compared with database sequences using the BLASTN and BLASTX programs. We present here the characterization of a cDNA clone (761-2) from YAC 761B5 which shows high homology with several human ESTs (98–100% identity) and mouse EST W10530 (81.4%), respectively. Clone 761-2 was found to reside on chromosome 21 at the expected location in 21q22.3 by both FISH analysis and Southern blot analysis with a human-mouse hybrid containing chromosome 21 as the only human chromosome (data not shown). When clone 761-2 was used to probe filters containing mRNA from several adult and fetal human tissues, two bands of approximately 2.2 and 2.7 kb were detected in most of the tissues investigated (Fig. 1); the highest levels of expression were found in heart, pancreas, prostate and placenta. Lower expression was found in other tissues including brain (Fig. 1), where spinal cord and medulla exhibited the highest levels (not shown). The cDNA corresponding to the 2.2 kb band was isolated (clone 2.2A) and characterized. The nucleotide and deduced protein sequences of this clone are presented in Fig. 2. The sequence shows the presence of an ORF of 518 amino acids based on the first ATG at position 109 (the sequence around the first ATG has a good homology to the Kozak consensus sequence) flanked by a 5′ UTR of 108 bp and a 3′ UTR of 197 bp respectively.

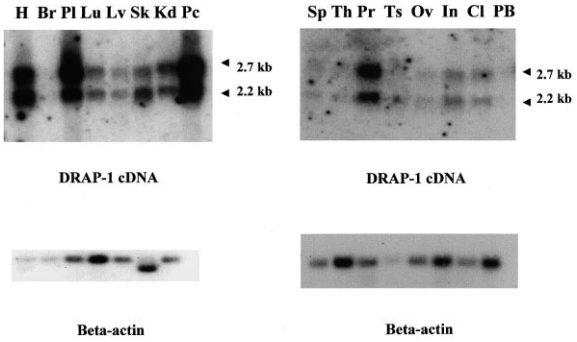

Fig. 1. Northern analysis of cDNA clone 761-2: total RNA (25 μg) from several human tissues was separated in a denaturing formaldehyde gel, transferred to positively charged nylon membranes and probed with random-primed labeled clone 761-2 DNA. Two hybridizing bands of approximate molecular weight of 2.2 and 2.7 kb were observed in most tissues analyzed (top panels). Normalization of RNA content in the blots was achieved following hybridization of the same filters with a β-actin probe (bottom panels). The tissue's nomenclature is as follows: H: heart; Br: brain; Pl: placenta; Lu: lung; Lv: liver; Sk: skeletal muscle; Kd: kidney; Pc: pancreas; Sp: spleen; Th: thymus; Pr: prostate; Ts: testis; Ov: ovary; In: intestine; Cl: Colon; PB: peripheral blood cells.

An initial similarity search against the SWISS-PROT protein database using the amino acid sequence encoded by clone 2.2A revealed a central core, corresponding to residues 82–329, with up to 32.2% identity to members of the A1 family of aspartyl endopeptidases (lowest BLAST E-value = 1e-26). Significantly, the amino acid residues comprising the two catalytic sites found in all mammalian aspartic proteases (Asp-Ser/Thr-Gly-Ser/Thr) are perfectly conserved in the cDNA clone, at positions 110–113 and 303–306, respectively. Furthermore, all the residues immediately adjacent to these sites are com-

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Fig. 2. Sequence of cDNA clone 2.2A: The 1881 bp sequence of the insert of clone 2.2A is shown. The ATG start codon lies at position 109 and is followed by a 518 residues ORF terminating with the stop codon TGA at position 1663. The two catalytic triads (DTG and DGS) are boxed. Two potential *N*-glycosylation sites are circled. A putative transmembrane domain is underlined.

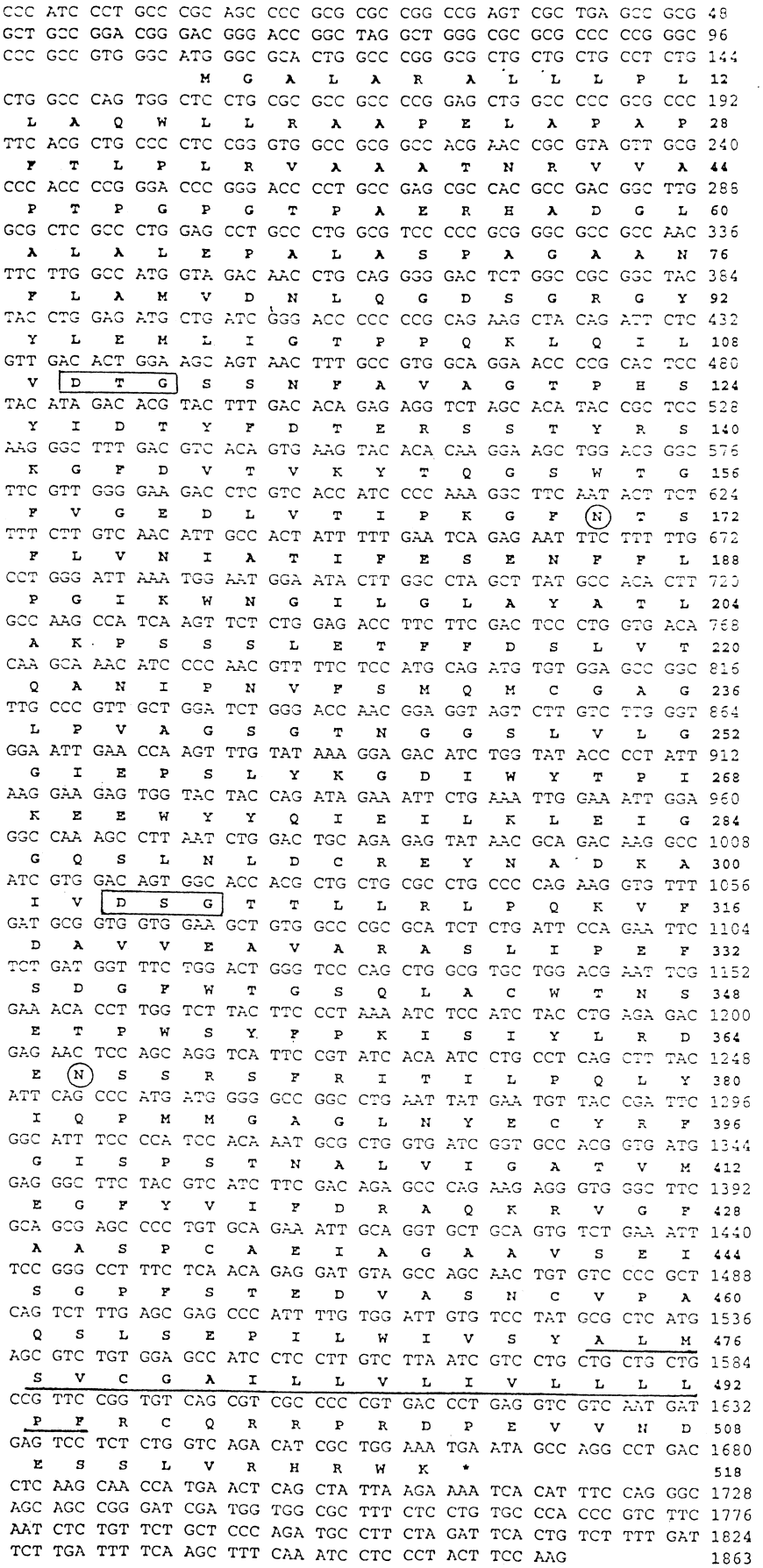
CCC ATC CCT GCC CGC AGC CCC GCG CGC CGG CCG AGT CGC TGA GCC GCG 48
GCT GCC GGA CGG GAC GGG ACC GGC TAG GCT GGG CGC GCG CCC CCG GGC 96
CCC GCC GTG GGC ATG GGC GCA CTG GCC CGG GCG CTG CTG CTG CCT CTG 144
M G A L A R A L L L P L 12
CTG GCC CAG TGG CTC CTG CGC GCC GCC CCG GAG CTG GCC CCC GCG CCC 192
L A Q W L L R A A P E L A P A P 28
TTC ACG CTG CCC CTC CGG GTG GCC GCG GCC ACG AAC CGC GTA GTT GCG 240
F T L P L R V A A A T N R V V A 44
CCC ACC CCG GGA CCC GGG ACC CCT GCC GAG CGC CAC GCC GAC GGC TTG 288
P T P G P G T P A E R H A D G L 60
GCG CTC GCC CTG GAG CCT GCC CTG GCG TCC CCC GCG GGC GCC GCC AAC 336
A L A L E P A L A S P A G A A N 76
TTC TTG GCC ATG GTA GAC AAC CTG CAG GGG GAC TCT GGC CGC GGC TAC 384
F L A M V D N L Q G D S G R G Y 92
TAC CTG GAG ATG CTG ATC GGG ACC CCC CCG CAG AAG CTA CAG ATT CTC 432
Y L E M L I G T P P Q K L Q I L 108
GTT GAC ACT GGA AGC AGT AAC TTT GCC GTG GCA GGA ACC CCG CAC TCC 480
V D T G S S N F A V A G T P H S 124
TAC ATA GAC ACG TAC TTT GAC ACA GAG AGG TCT AGC ACA TAC CGC TCC 528
Y I D T Y F D T E R S S T Y R S 140
AAG GGC TTT GAC GTC ACA GTG AAG TAC ACA CAA GGA AGC TGG ACG GGC 576
K G F D V T V K Y T Q G S W T G 156
TTC GTT GGG GAA GAC CTC GTC ACC ATC CCC AAA GGC TTC AAT ACT TCT 624
F V G E D L V T I P K G F N T S 172
TTT CTT GTC AAC ATT GCC ACT ATT TTT GAA TCA GAG AAT TTC TTT TTG 672
F L V N I A T I F E S E N F F L 188
CCT GGG ATT AAA TGG AAT GGA ATA CTT GGC CTA GCT TAT GCC ACA CTT 720
P G I K W N G I L G L A Y A T L 204
GCC AAG CCA TCA AGT TCT CTG GAG ACC TTC TTC GAC TCC CTG GTG ACA 768
A K P S S S L E T F F D S L V T 220
CAA GCA AAC ATC CCC AAC GTT TTC TCC ATG CAG ATG TGT GGA GCC GGC 816
Q A N I P N V F S M Q M C G A G 236
TTG CCC GTT GCT GGA TCT GGG ACC AAC GGA GGT AGT CTT GTC TTG GGT 864
L P V A G S G T N G G S L V L G 252
GGA ATT GAA CCA AGT TTG TAT AAA GGA GAC ATC TGG TAT ACC CCT ATT 912
G I E P S L Y K G D I W Y T P I 268
AAG GAA GAG TGG TAC TAC CAG ATA GAA ATT CTG AAA TTG GAA ATT GGA 960
K E E W Y Y Q I E I L K L E I G 284
GGC CAA AGC CTT AAT CTG GAC TGC AGA GAG TAT AAC GCA GAC AAG GCC 1008
G Q S L N L D C R E Y N A D K A 300
ATC GTG GAC AGT GGC ACC ACG CTG CTG CGC CTG CCC CAG AAG GTG TTT 1056
I V D S G T T L L R L P Q K V F 316
GAT GCG GTG GTG GAA GCT GTG GCC CGC GCA TCT CTG ATT CCA GAA TTC 1104
D A V V E A V A R A S L I P E F 332
TCT GAT GGT TTC TGG ACT GGG TCC CAG CTG GCG TGC TGG ACG AAT TCG 1152
S D G F W T G S Q L A C W T N S 348
GAA ACA CCT TGG TCT TAC TTC CCT AAA ATC TCC ATC TAC CTG AGA GAC 1200
E T P W S Y F P K I S I Y L R D 364
GAG AAC TCC AGC AGG TCA TTC CGT ATC ACA ATC CTG CCT CAG CTT TAC 1248
E N S S R S F R I T I L P Q L Y 380
ATT CAG CCC ATG ATG GGG GCC GGC CTG AAT TAT GAA TGT TAC CGA TTC 1296
I Q P M M G A G L N Y E C Y R F 396
GGC ATT TCC CCA TCC ACA AAT GCG CTG GTG ATC GGT GCC ACG GTG ATG 1344
G I S P S T N A L V I G A T V M 412
GAG GGC TTC TAC GTC ATC TTC GAC AGA GCC CAG AAG AGG GTG GGC TTC 1392
E G F Y V I F D R A Q K R V G F 428
GCA GCG AGC CCC TGT GCA GAA ATT GCA GGT GCT GCA GTG TCT GAA ATT 1440
A A S P C A E I A G A A V S E I 444
TCC GGG CCT TTC TCA ACA GAG GAT GTA GCC AGC AAC TGT GTC CCC GCT 1488
S G P F S T E D V A S N C V P A 460
CAG TCT TTG AGC GAG CCC ATT TTG TGG ATT GTG TCC TAT GCG CTC ATG 1536
Q S L S E P I L W I V S Y A L M 476
AGC GTC TGT GGA GCC ATC CTC CTT GTC TTA ATC GTC CTG CTG CTG CTG 1584
S V C G A I L L V L I V L L L L 492
CCG TTC CGG TGT CAG CGT CGC CCC CGT GAC CCT GAG GTC GTC AAT GAT 1632
P F R C Q R R P R D P E V V N D 508
GAG TCC TCT CTG GTC AGA CAT CGC TGG AAA TGA ATA GCC AGG CCT GAC 1680
E S S L V R H R W K * 518
CTC AAG CAA CCA TGA ACT CAG CTA TTA AGA AAA TCA CAT TTC CAG GGC 1728
AGC AGC CGG GAT CGA TGG TGG CGC TTT CTC CTG TGC CCA CCC GTC TTC 1776
AAT CTC TGT TCT GCT CCC AGA TGC CTT CTA GAT TCA CTG TCT TTT GAT 1824
TCT TGA TTT TCA AGC TTT CAA ATC CTC CCT ACT TCC AAG 1863

Fig. 3. Alignment of the open reading frame predicted from the cDNA sequence of the human DRAP/BACE2 clone to the sequence of human BACE-1 protein (GenBank accession number AF190725). Numbers indicate amino acid positions. Identical amino acids are shaded in grey and homologous ones (GES scale) are outlined in black. The boxes including DRAP-1 residues 107 to 118 and 300 to 311 indicate the amino acids corresponding to the canonical aspartic protease PROSITE pattern (PROSITE accession number PS00141), with the conserved signature sequences D T/S G T/S marked. Predicted signal peptide cleavage sites and transmembrane domains are indicated by red arrows and boxed in cyan, respectively. Putative *N*-linked glycosylation sites are marked with pale dots, lumenal cysteine residues are indicated by dark dots.

patible with the PROSITE pattern defined for the aspartic protease class (accession number PS00141). Taken together, these findings prompted us to give this novel gene the name DRAP, the gene nomenclature has approved the symbol BACE2 for this gene).

The presence of a signal peptide (residues 1 to 20) predicting cleavage between Ala-20 and Ala-21 was indicated by analysis using a specific algorithm for the prediction of signal peptide sequences (web site http://genome.cbs.dtu.dk); however no homology was observed between this peptide and signal peptides of other aspartic proteases. This finding is not totally unexpected, as it is known that signal peptides in general have a low level of primary sequence conservation.

Although most aspartic proteases have activation peptides with a similar sequence and structural homology to one another, we failed to observe any sequence of DRAP/BACE2 that could be reminiscent of such conserved sequences. Two potential *N*-glycosylation sites at amino acid positions 170 and 366 were also noted in the coding region of clone 2.2A.

Further sequence analysis also predicted the presence of hydrophobic domains suitable to serve as membrane spanning regions: submission of the protein sequence to two different membrane spanning prediction algorithms (TMPred [23]. (http://www.isrec.isb-sib.ch/software/TMPRED_form.html; moment transmembrane helix prediction at the UCLA-DOE Fold-Recognition server [21]. (http://fold.doe-mbi.ucla.edu/Home), yielded a number of domains among which the absolute highest score was achieved by the segment enclosed between Ala at position 474 and Phe at position 494.

The latter 21 amino acid stretch is predicted to form a transmembrane domain that would confer to the whole protein the orientation of a type 1a integral membrane protein [11]. Such membrane topology is suggested by the presence of several basic arginine residues located at the C-terminal side of the putative transmembrane domain [24,25]. Thus, considering the location of this second hydrophobic domain (near the C-terminus of the ORF), most of the protein would be expected to translocate across the ER membrane following translation of the putative aspartic protease-encoding mRNA, leaving a C-terminal 24 amino acid untranslocated stretch on the cytoplasmic side.

Furthermore once the sequence of BACE-1 (Fig. 3) became available it was obvious that DRAP/BACE2 and BACE-1 are much more similar to one another than to any other member of the aspartic protease family; the two proteins are in fact 45% identical and 71% homologous. As indicated in Fig. 3, the positions of the predicted signal peptide cleavage sites, of the putative transmembrane regions, of one of the putative glycosylation sites and of the lumenal cysteine residues are conserved between the two proteins.

The prediction of such transmembrane domain in DRAP/BACE2 was a rather unexpected finding, as most aspartic proteases described so far in vertebrates are known to be secreted proteins. We therefore assessed whether sequences with similar properties also existed in other vertebrate species. To this end, we decided to clone the mouse homologue of DRAP/BACE2 by screening a mouse skeletal muscle cDNA library with EST W10530, which was previously shown to bear a significant homology with DRAP/BACE2; a partial cDNA covering the most 3′ end of the sequence corresponding to amino acid 264–518 of the human sequence was obtained. Both at the nucleotide and amino acid level a remarkable identity is present between the two sequences. In

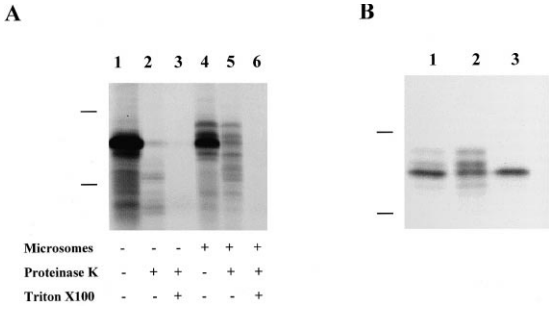

Fig. 4. SDS–PAGE analysis of in vitro translated DRAP/BACE2. A: the DRAP/BACE2 coding region was cloned into the expression vector pSP64Tnew as described in Section 2, the resulting construct was transcribed in vitro and the purified RNA was translated in vitro in the presence of [^{35}S]methionine; the reaction products (5 μl per lane) were then analyzed on a 15% denaturing polyacrylamide gel. In vitro translation reactions were performed in the presence and absence of canine pancreatic microsomal membranes, and aliquots of the reactions were further treated with proteinase K and Triton X-100 (as indicated in the diagram below the picture) under the conditions described in Section 2. The two vertical lines on the left side of the picture represent the position of the protein molecular weight marker bands corresponding to 46 and 66 kDa. B: In vitro translated DRAP was treated with a solution of 100 mM sodium carbonate at pH 11.5 as described in Section 2 to disrupt the microsomal membranes and the membrane-containing fraction was harvested by centrifugation at 100 000 × *g* for 30 min. Aliquots from the membrane fraction (lane 2) and the supernatant (lane 3) were then analyzed by SDS–PAGE together with a sample of protein that was not fractionated (lane 1). The two vertical lines on the left side of the picture represent the position of the protein molecular weight marker bands corresponding to 46 and 66 kDa.

particular, the predicted transmembrane domain (spanning amino acids 474–494 in the human sequence) is well conserved in the mouse cDNA clone, since over a stretch of 21 amino acids only two substitutions are present at positions 488 (Val-Leu) and 494 (Phe-Leu), respectively (data not shown). The C-terminal catalytic site (DSG) was also perfectly conserved in the murine cDNA. Thus a gene encoding a putative transmembrane aspartic protease seems to exist also in the mouse.

3.2. In vitro synthesis of DRAP/BACE2

In order to assess whether the DRAP predicted transmembrane domains are functional, cDNA clone 2.2A was in vitro transcribed and the RNA was translated with wheat germ extracts; SDS–PAGE analysis of the in vitro translation products revealed a major polypeptide with the expected M_r of 56 kDa (Fig. 4A, lane 1). When canine pancreatic microsomal membranes were included in the translation reaction, additional polypeptides of 58 and 60 kDa were observed, presumably representing glycosylated molecules (Fig. 4A, lane 4). The appearance of polypeptides migrating faster than the 56 kDa polypeptide was occasionally observed, and their nature was not further investigated.

Proteinase K treatment of the translation products showed that while the vast majority of the 56 kDa polypeptide synthesized in the absence of microsomal membranes was susceptible to proteolytic attack (Fig. 4A, lane 2), the 58 and 60 kDa polypeptides synthesized in the presence of microsomes were largely resistant, indicating that they had been translocated into the microsomal vesicles (Fig. 4A, lane 5). A small amount of the 56 kDa protein was also consistently observed after proteinase K treatment (lane 5), and may represent either translocated protein that has not yet been glycosylated, or a small residue of the untranslocated 56 kDa band that escaped degradation, or a mixture of both; we favor the former explanation on the basis of the results obtained following sodium carbonate treatment of the in vitro translated protein (see below).

In agreement with the previous results, all translation products were quantitatively degraded when a detergent was included in the proteinase K digestion reaction. (Fig. 4A, lanes 3 and 6). Moreover, in order to distinguish whether the putative aspartic protease is an integral membrane protein embedded in the lipid bilayer or a peripheral protein held in association with the membrane by electrostatic interactions, we challenged the in vitro translated, microsome associated protein with a sodium carbonate solution at pH 11; under these strong alkaline conditions, the three ^{35}S-labeled bands representing the ER-translocated, glycosylated protein were coprecipitated with the microsomal membranes following ultracentrifugation (Fig. 4B), suggesting that the protein under scrutiny behaves in vitro like an integral membrane-spanning protein.

Since addition of a single *N*-linked chain normally causes an apparent increase in M_r of about 2 kDa and since the primary sequence of the putative translation product contains two potential *N*-glycosylation sites, we investigated the possibility that the 58 and 60 kDa products could represent glycosylated forms of the 56 kDa polypeptide. Treatment with endoglycosidase H (Endo H), which removes N-linked, high mannose oligosaccharide chains, resulted in the disappearance of the 60 kDa polypeptide, indicating that it indeed represents a glycosylated form of the 56 kDa polypeptide (Fig. 5A, lane 3). However, Endo H treatment did not apparently affect the 58 kDa polypeptide. Since some glycan chains might be inaccessible to the action of Endo H, we used two alternative approaches to determine whether this latter polypeptide was indeed glycosylated. N-linked glycosylation can be inhibited in vitro by supplementing the translation reaction with an acceptor tripeptide (acetyl)-Asn-Tyr-Thr-(amide) which competes with the translated protein for the transfer of the oligosaccharide chain. As shown in Fig. 5B (lane 3), both the 58 and 60 kDa protein bands observed in the presence of microsomal membranes were not observed when the tripeptide was included in the translation reaction, thus providing strong evidence that both bands indeed represent different glycoforms of the in vitro synthesized polypeptide. The glycosylated nature of the two polypeptides was further confirmed by including an inhibitor of ER glucosidase I in the translation reaction. In the presence of this inhibitor, the removal of the three glucose residues from the N-linked oligosaccharides does not occur, resulting in a decreased mobility of the glycosylated polypeptides. As expected, both bands were supershifted in the presence of such a inhibitor (Fig. 5C, lane 3), thus providing definitive evidence that both were glycosylated.

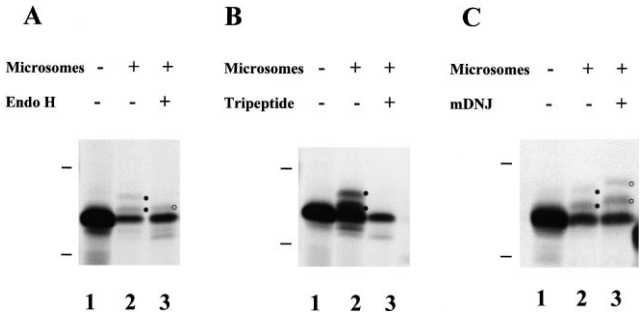

Fig. 5. DRAP/BACE2 is a glycoprotein. A: DRAP/BACE2 was translated in vitro as described before in the presence and absence of microsomal membranes; an aliquot of the reaction was then treated with Endo H as described in Section 2 before being analyzed by SDS–PAGE. B: As an independent evidence of DRAP-1 glycosylation, in vitro translation was performed in the presence of the glycosylation inhibitor (acetyl)-Asn-Tyr-Thr-(amide) acceptor tripeptide. C: An independent in vitro translation reaction was also carried out in the presence of the β-glucosidase inhibitor *N*-methyldeoxynojirimycin. Asterisks in panels A, B and C, lanes 2 indicate the position of the 58 and 60 kDa bands observed in the presence of microsomal membranes. Open circles in panel A, lane 3 and panel C, lane 3 indicate the Endo H resistant form of DRAP/BACE2 and the two supershifted bands observed in the presence of *N*-methyldeoxynojirimycin, respectively. The two short horizontal lines on the left side of the picture represent the position of the protein molecular weight marker bands corresponding to 46 and 66 kDa.

To confirm the in vitro evidence for DRAP behaving as a transmembrane protein, we transfected HeLa cells with an expression vector encoding DRAP tagged at its C-terminal with a human c-myc epitope. Immunofluorescence analysis of Triton X permeabilized cells with a mouse conjugate antibody system directed against the c-myc epitope indicated that at least some of the protein reaches the plasma membrane whereas most is retained in an intracellular compartment, possibly the Golgi (not shown).

4. Discussion

Down syndrome (DS) is the most frequent human aneuploidy, occurring in 1 in 600 to 1 in 800 live births and is

caused by the triplication of chromosome 21 [26]. The symptoms of this syndrome are considered to occur through damage caused by overexpression of normal gene products encoded by genes on this chromosome. The elevated levels of these proteins are thought to impair an unknown set of genetic pathways which ensures normal cellular functioning [26].

A Down syndrome critical region (DSCR) on 21q has been defined on the basis of data obtained from patients with partial triplication of chromosome 21 [26]. A consistent feature of Down syndrome in middle aged patients is the deposition in the brain of amyloid plaques resembling those observed in Alzheimer disease [26]. β-secretases are important enzymes in the generation of amyloid plaques, as they cleave APP to release amyloidogenic peptides [27].

The discovery in chromosome 21 of a gene encoding a membrane bound, putative aspartyl-protease highly homologous to β-secretase, is an intriguing finding that could have potential implications for the pathogenesis of one of the major neurological findings of DS patients, namely the Alzheimer disease-related manifestations. DRAP/BACE2 might represent a second type of β-secretase as indicated both by the high level of homology to BACE-1 (Fig. 3) and by its transmembrane location (Fig. 5), a typical feature of BACE but not of other aspartic proteases. Indeed it is known that although AβAsp1 is the most prominent species formed upon β-secretase cleavage, other amino terminal Aβ peptides, like Val-3, Glu-11 and Ile-6, are also generated [28–31]. BACE-1 cleaves at Asp-1 and Glu-11 but not at other positions [10]. Interestingly, the NH2-terminal truncated Aβ forms (in particular pyroglutamate 3–42) increase with age in Down's syndrome brain, and have a crucial role in promoting Aβ aggregation in amyloid fibrils [28–32].

Work is in progress to assess the cleavage specificity of DRAP/BACE2 upon APP. If DRAP/ BACE2 should indeed turn out to be a second β-secretase this would suggest the intriguing hypothesis that increased dosage of both the genes encoding the processing enzyme (DRAP/BACE2) and the substrate (APP) could underlie the development of amyloid plaques in Down patients.

During completion of this manuscript Saunders et al. and Louis et al. [33,34] compared the sequence reported in this paper (DRAP-1/BACE2 accession number AF050171 deposited February 28, 1998) with BACE discussing their similarities and named it BACE2.

The accession numbers of the human and mouse DRAP cDNA sequences are AF050171 and AF051150, respectively.

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