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Primary structure of a novel ABC transporter with a chromosomal localization on the band encoding the multidrug resistance-associated protein

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Abstract Complementary DNA clones encoding a novel protein, ABC-C, with the typical structural features of the ABC transporter family were identified in a human medullary thyroid carcinoma cell line. The transporter consists of 1704 amino acid residues with two homologous repeats, each harboring six putative transmembrane helices and an ATP-binding cassette motif. The mRNA is expressed highest in normal lung, but also in varying amounts in other tissues and in C-cell carcinoma. The ABC-C gene is mapped on chromosome 16p13.3, in close physical proximity to another ABC transporter, the multidrug resistance-associated protein. This related protein is assumed to confer resistance to chemotherapeutic drugs in small cell lung carcinoma. The genomic clustering of both transporters, typical also for other members of the ABC family, supports the notion that ABC-C may be involved in development of resistance to xenobiotics.

Key words: ABC transporter; Chemotherapy; Multidrug resistance; Thyroid carcinoma

1. Introduction

The resistance of malignant tumors to chemotherapy is considered to be partly due to overexpression of transmembrane transport proteins. The human multidrug resistance protein (MDR) and probably the multidrug resistance-associated protein (MRP) utilize the energy of ATP hydrolysis to pump chemotherapeutic drugs out of the cells. Both proteins are members of the ATP-binding cassette (ABC) superfamily, which are encoded by one of the largest and diverse gene families (reviewed in [1,2]). Many ABC transporters are of considerable clinical significance not only for conferring resistance to chemotherapeutics, but also as drug targets. The receptor for sulfonylurea is a member of the ABC transporter family which binds the orally active antidiabetics and regulates the opening of a potassium channel [3]. This receptor is mutated in patients with persistent hyperinsulinemic hypoglycemia of infancy [4]. Mutation of genes encoding ABC transporters are involved in diverse hereditary diseases such as cystic fibrosis, adrenoleukodystrophy and in defects in antigen processing [5–7].

Gene transfer experiments with MDR1 cDNA have demonstrated that the expression of P-glycoprotein is sufficient to transfer the multidrug resistance phenotype to drug-sensitive cells [8]. P-glycoprotein functions as an ATP-dependent efflux pump that reduces the intracellular accumulation of cytotoxic agents. It interacts directly with a variety of antineoplastic drugs and transports them out of the cells.

The widespread occurrence of drug resistance cannot solely be explained by the overexpression of the P-glycoprotein. In thyroid cancer, a very low frequency of MDR1 expression was found by the RT-PCR technique [9]. The expression of the MDR1-related MRP transporter was highest in anaplastic carcinoma, in which tumor MRP was present in over 50% of the tissues [10]. However, other carcinomas such as papillary, follicular or medullary carcinoma had a very low or no expression of the MRP transporter. In addition, a human medullary thyroid carcinoma TT cell line did not express the P-glycoprotein, although the effects of chemotherapeutic drugs were potentiated by verapamil and cyclosporin A [11].

In order to elucidate the existence of additional ABC transporters which may contribute to drug resistance in thyroid cancer we used a human medullary thyroid carcinoma cell line for a cDNA cloning approach. A new member of the ABC transporter family could be identified. It shows the typical structural features of these transmembrane proteins, such as the putative four domain topology and the conserved motifs of the ATP-binding cassettes. Since the cDNA clone originated from human medullary thyroid C-cells it is designated as ABC-C.

2. Materials and methods

2.1. Isolation of RNA and Northern blot analysis

Total RNA from a human C-cell carcinoma was isolated by the guanidinium thiocyanate method and poly(A) RNA was separated by oligo(dT) cellulose chromatography (Poly(A) Quik mRNA Isolation Kit, Stratagene). Poly(A) RNA from hMTC cells was purified using the Fast Track Kit (Invitrogen). Poly(A) RNA from hMTC cells and from the C-cell carcinoma was fractionated on a 1.2% agarose gel, transferred to nylon membranes (Pall) and hybridized under high stringency. The human multiple tissue Northern Blot was obtained from Clontech. The random primed labeled 1308 bp *Sac*I fragment was used as probe; this region shares no significant homology with other sequences of ABC transporters.

2.2. cDNA library construction

Poly(A) RNA from hMTC cells was reverse transcribed and double-strand cDNA was synthesized using the Superscript plasmid system (Gibco BRL Life Technologies). The cDNA fragments were ligated with *Bst*XI/*Eco*RI adaptors (Invitrogen). The cDNA was size-fractionated on a low melting agarose gel (FMC SeaPlaque GTG agarose, BIOzym) and only gel slices containing fragments >4000 bp were excised and digested with GELase (BIOzym). The cDNA was ligated into the *Bst*XI site of the pcDNAII vector (Invitrogen) and transformed into the *E. coli* XL1-Blue MRF′ strain (Stratagene). A degenerate oligonucleotide derived from the conserved sequence motif of ABC transporters was used to screen the library under low stringency two times. Several clones hybridized with different autoradiographic signal strength to the oligonucleotide probe. Only clones

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Abbreviations: ABC, ATP-binding cassette; ATS, active transport signature; hMTC, human medullary thyroid carcinoma

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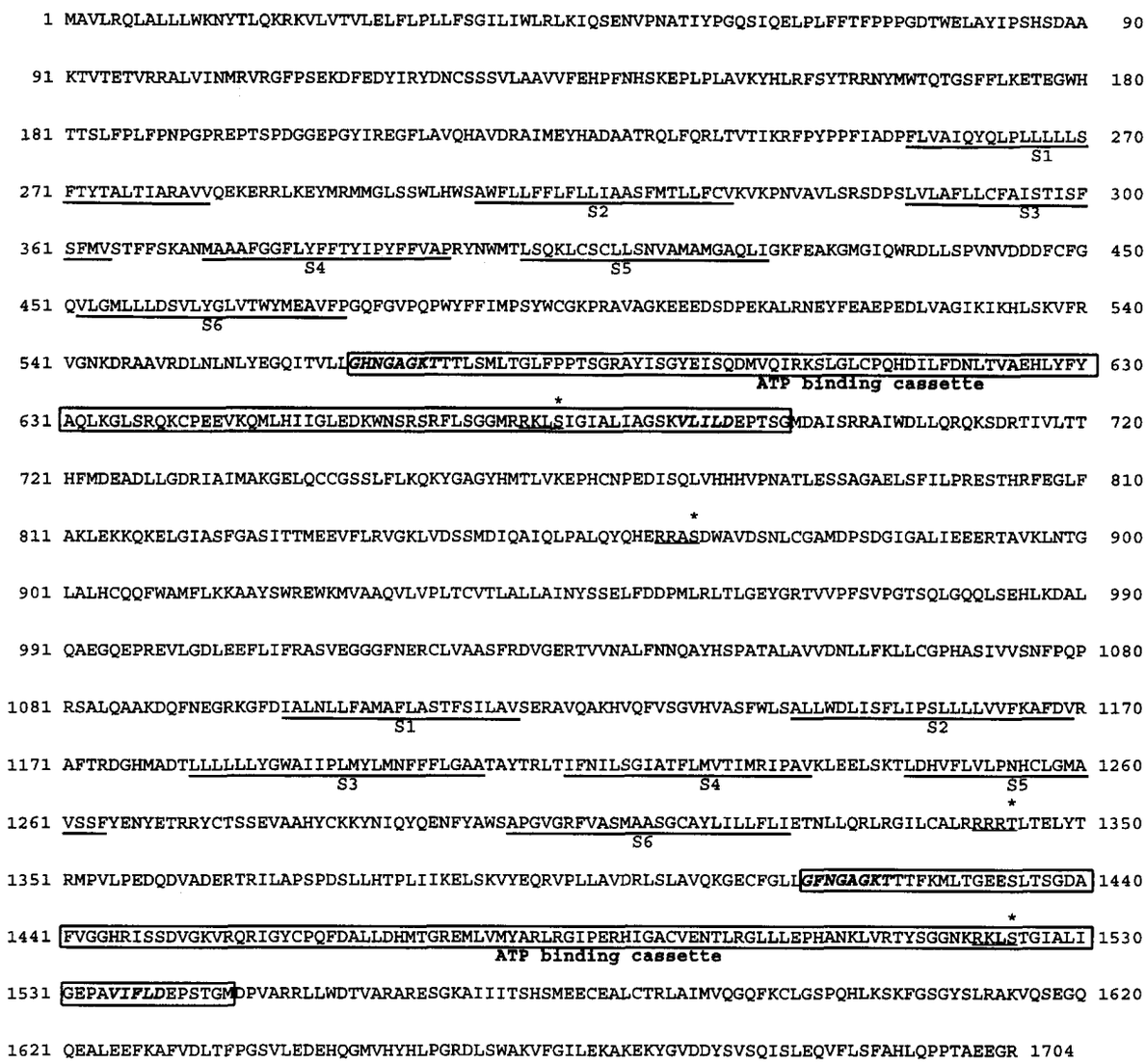

Fig. 1. Primary structure of ABC-C. The amino acid sequence was deduced from its cDNA. The suggested locations of the transmembrane segments are designated by horizontal lines. The conserved motifs of the ATP-binding cassettes are indicated by the open bars. Potential phosphorylation sites by cAMP kinase are underlined and marked with asterisks.

which were positive in both rounds of screening were analyzed further by restriction digestion and by sequencing. Two of the 20 analyzed clones had an almost identical restriction analysis pattern.

2.3. Sequence analysis

Sequencing was performed using the Thermo Sequenase cycle sequencing protocol (Amersham) in combination with the A.L.F. DNA sequencer (Pharmacia Biotech). pABC18 was digested with different restriction endonucleases and the resulting cDNA fragments were subcloned into pUC18. Fluorescent labelled M13 universal and reverse primers were used for sequencing both strands. Sequence stretches with no available subclone were analyzed by specific primers and by the dideoxy chain termination method on double-stranded templates (USB Sequenase 2.0). The sequence is available under accession number X97187 at the EMBL nucleotide sequence database.

3. Results and discussion

3.1. Primary structure

Screening of a hMTC cDNA library revealed two positive independent clones, pABC18 and pABC52. The complete nucleotide sequence of pABC18 consists of 6246 bp, pABC52 is 5 bp longer at the 3′-untranslated region, but 54 bp shorter at the 5′-untranslated region. The identity of both clones was proved by a detailed restriction analysis and by sequencing of several subclones. The deduced open reading frame of pABC18 comprises 5112 bp encoding a 1704 amino acid protein with a calculated molecular mass of 191 360 Da (Fig. 1). The putative translational start codon is the first in-frame ATG codon of the open reading frame. Several termination codons upstream of the initiation site of translation indicate that both clones represent full-length cDNAs. The region directly upstream of the translational start site resembles the consensus sequence for eukaryotic initiation sites with an A base at position −3 and a G base at +4 relative to the start codon. The 3′-untranslated region contains one consensus polyadenylation signal very close to the 3′-end of the cDNA fragment, but both clones have no poly(A) tail.

Hydropathy analysis predicts a structure reminiscent of that of the largest ABC transporters, such as multidrug resistance

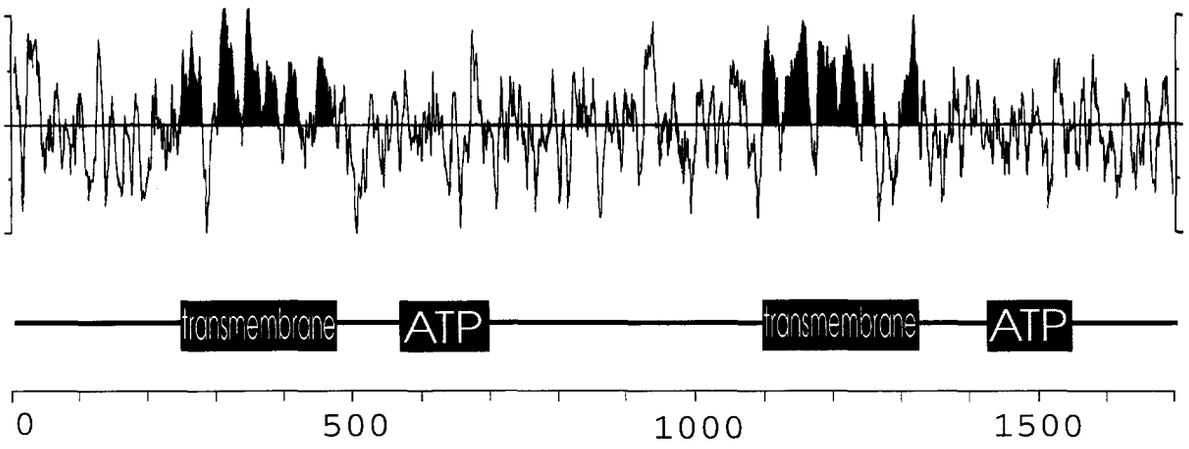

Fig. 2. Hydropathy analysis according to Kyte and Doolittle [18]. The curve is the average of a residue specific hydrophobicity index over a window of nine residues. The line in the upper half indicates hydrophobic regions and that in the lower half hydrophilic regions. Putative transmembrane segments are black colored. Lower part: Schematic representation of the location of the putative transmembrane regions and of the ATP-binding cassettes.

P-glycoprotein or CFTR (Fig. 2). ABC-C shows the typical domain composition consisting of two repeats each with six transmembrane segments and an ATP binding cassette motif. Assuming an intracellular N-terminus, as is the case for all other mammalian ABC transporters, the ABC-C transporter consists of three cytoplasmic regions and two transmembrane regions. Only some short sequence stretches connecting the transmembrane segments are located extracellularly. However, the intracellular loop between both repeats contains two additional hydrophobic regions and it is possible that they are also embedded in the membrane. An extra hydrophobic segment has also been identified in the ABC1 and ABC2 transporters which have been identified in a murine macrophage cell line [12]. The murine ABC1 and ABC2 transporter show the closest relationship to ABC-C. Comparative sequence analysis between ABC-C and the mouse ABC1 transporter reveals 40% identical and 62% similar amino acids. However, this low degree of similarity indicates that ABC-C does not represent the corresponding human protein of one of the murine transporters.

The multidrug resistance protein MDR1 [13] and its associated protein MRP [14] are 50% and 44% homologous to ABC-C, respectively. MDR1 and MRP are only distantly related to each other. Sequence comparisons with other mem-

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ABC-C (N) AVRDLNLNLYEGQITVLLGHNGAGKTTTLSMLTGLFPPTSGRAYISGYEISQDMVQ-IRKSLGLCPQHDILFDNLTVAEHLYFYAQLKGLSRQKC  641
mABC1 (N) AVDGLALNFYEGQITSFLGHNGAGKTTTMSILTGLFPPTSGTAYILGKDIRSEMSS-IRQNLGVCPQHNVLFDMLTVEEHIWFYARLKGLSEKHV  948
hMDR1 (N) ILKGLNLKVQSGQTVALVGNSGCGKSTTVQLMQRLYDPTEGMVSVDGQDIRTINVRFLREIIGVVSQEPVLFATTIAENIRYGRENVTMDEIEKA  503
hMRP  (N) TLNGITFSIPEGALVAVVGQVGCGKLSLLSALLAEMDKVEGHVAIKGS----------VAYVPQQAWIQNDSLRENILFGCQLEEPYYRSVI  741
                                    WA

ABC-C (N) ---PEEVKQMLHIIGL-EDKWNSRSRFLSGGMRRKLSIGIALIAGSKVLILDEPTSGMDAISRRAIWDLLQRQKSDRTIVLTTHFMDEADLLGDR  732
mABC1 (N) ---KAEMEQMALDVGLPPSKLKSKTSQLSGGMQRKLSVALAFVGGSKVVILDEPTAGVDPYSRRGIWELLLKYRQGRTIILSTHHMDEADILGDR 1040
hMDR1 (N) VKEANAYDFIMKLPHKFDTLVGERGAQLSGGQKQRIAIARALVRNPKILLLDEATSALDTESEAVVQVALDKARKGRTTIVIAHRLSTVRNADVI  591
hMRP  (N) QACALLPDLEIL-PSGDRTEIGEKGVNLSGGQKQRVSLARAVYSNADIYLFDDPLSAVDAHVGKHIFENVIGPKGMLKNKTRILVTHSMSYLPQV  835
                                    ATS                 WB

ABC-C (C) AVDRLSLAVQKGECFGLLGPNGAGKTTTFKMLTGEESLTSGDAFVGGHRISSDVGK-VRQRIGYCPQFDALLDHMTGREMLVMYARLRGIPERHI 1491
mABC1 (C) AVDRICIGIPPGECFGLLGVNGAGKSTTFKMLTGDTPVTRGDAFLNKNSILSNIHE-VHQNMGYCPQFDAITELLTGREHVEFFALLRGVPEKEV 1961
hMDR1 (C) VLQGLSLEVKKGQTLALVGSSGCGKSTVVQLLERFYDPLAGKVLLDGKEIKRLNVQWLRAHLGIVSQEPILFDCSIAENIAYGDNSRVVSQEEIV 1146
hMRP  (C) VLRHINVTINGGEKVGIVGRTGAGKSSLTLGLFRINESAEGEIIIDGINIAKIGLHDLRFKITIIPQDPVLFSGSLRMNL---DPFSQYSDEEVW 1403
                                    WA

ABC-C (C) ------GACVENTLRGLLLEPHANKLVRTYSGGNKRKLSTGIALIGEPAVIFLDEPSTGMDPVARRLLWDTVARARESGKAIIITSHSMEECEAL 1580
MABC1 (C) ------GKFGEWAIRKLGLVKYGEKYASNYSGGNKRKLSTAMALIGGPPVVFLDEPTTGMDPKARRFLWNCALSIVKEGRSVVLTSHSMEECEAL 2050
hMDR1 (C) RAAKEANIHAFIESLPNKYSTKVGDKGTQLSGGQKQRIAIARALVRQPHILLLDEATSALDTESEKVVQEALDKAREGRTCIVIAHRLSTIQNAD 1241
hMRP  (C) TSLELAHLKDFVSALPDKLDHECAEGGENLSVGQRQLVCLARALLRKTKILVLDEATAAVDLETDDLIQSTIRTQFEDCTVLTIAHRLNTIMDYT 1495
                                    ATS                 WB
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Fig. 3. Multialignment of the extended nucleotide-binding cassettes from ABC-C, ABC1, MDR1 and MRP. The amino-terminal (N) and carboxy-terminal (C) located sequences are aligned at the positions indicated. The conserved Walker A and B motifs (WA and WB) and the active transport family signature (ATS) characteristic of nucleotide-binding folds of ABC transporters are indicated.

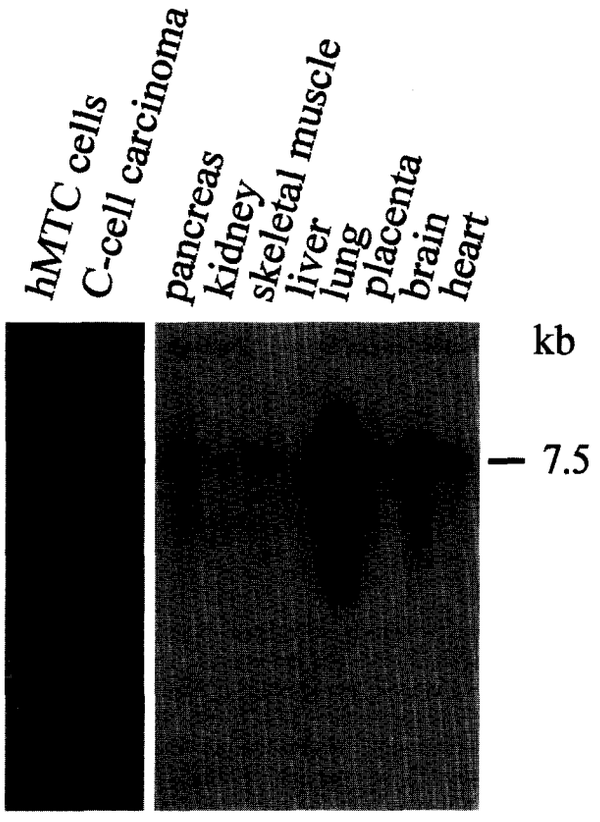

Fig. 4. Northern blot analysis of poly(A) RNA from hMTC cells, a C-cell carcinoma and different human tissues. Left part: 5 μg of poly(A) RNA isolated from hMTC cells and from a C-cell carcinoma was hybridized with an ABC-C specific probe. The film was exposed for 2 days. Right part: Autoradiograph of 2 μg of poly(A) RNA from normal human tissues. Time of exposure was 16 h.

bers of the ABC transporter family revealed similarity values below 45%.

The highest degree of homology resides in the two conserved motifs of the ATP-binding cassettes (Fig. 3). The sequence identity extends over the entire domains and is larger than within the two short stretches of the Walker motifs [1,15]. The Walker A motif corresponds to a glycine-rich loop known to be involved in phosphoryl transfer in many nucleotide-binding proteins. The active transport signature (ATS, Fig. 3) is located a certain distance upstream of the Walker B motif.

3.2. Database search and chromosomal mapping

The European Molecular Biology Laboratory and the TIGR human cDNA database were searched for similar sequences to ABC-C using different programs (Blastn, Blastp, Tblastn). Several expressed sequence tags from human brain and fetal lung libraries shared identical sequence stretches, but none of the partial clones extended more than several hundred base pairs. Therefore they are far from representing the full-length cDNA. At the genomic DNA level the database search revealed that part of ABC-C is 100% identical to clone exon trap b76, which was identified by exon trapping of the region involved in polycystic kidney disease (accession number L48760, Burn, T.C., Connors, T.D., Landes, G.M. and Klinger, K.W., unpublished). This exon has been mapped to chromosome 16p13.3. The identity over the entire exon sequence clearly demonstrates that the ABC-C transporter is encoded in the same chromosomal region. Recently, it was shown that the gene coding for the MRP is located at the same chromosome band p13.3 [14]. As already suggested by these authors additional genes on the same chromosomal band may be responsible for or contribute to the multidrug resistance phenotype of a lung cancer cell line. The close physical proximity of the gene locus indicates that ABC-C could be this candidate. Linkage of gene loci for members of the ABC transporter family can also be observed for the human CFTR and MDR1 proteins, which have been mapped to chromosome 7 at bands q31 and q21, respectively [16,17]. In situ hybridization using the murine ABC1 and ABC2 cDNAs as probes on human chromosome spreads identified the ABC1 and ABC2 genes on chromosome 9 region q22–q31 and q34, respectively [12]. This result and the low degree of sequence similarity support further the notion that ABC-C is not the human homolog of one of the murine proteins.

3.3. Expression of ABC-C

Northern blot analysis of mRNA from hMTC cells and from a solid C-cell carcinoma both show a single transcript of 7.5 kb (Fig. 4). The same transcript is present in different human tissues, although with variations in abundance. ABC-C is expressed highest in lung but is also clearly detectable in brain and pancreas, expression is lower in heart and skeletal muscle. An overnight exposure is sufficient to observe mRNA in these tissues. Prolonged autoradiography demonstrates that ABC-C transcripts of the same size can be identified also in placenta, kidney and liver at low amounts (not shown).

According to the expression of MRP, which is readily detectable in lung, ABC-C is most abundant in lung. Both the amounts of MRP and ABC-C mRNA in the normal lung are considerably higher than those detected in the cell lines from which they were initially isolated (for discussion see [12]). Some cell types in the lung or in the thyroid gland may be inherently drug-resistant because of an increased constitutive expression of MRP or ABC-C, respectively. In contrast, the expression pattern of MRP in other tissues is quite different from that of ABC-C. MRP transcripts for example are not observed in brain, placenta, kidney and liver.

The genomic clustering of ABC-C with MRP together with the conserved domain structure indicates that ABC-C may be functionally related and may act as an efflux pump for chemotherapeutic drugs. However, the distinct expression pattern of MDR, MRP and ABC-C suggests that their physiological roles may differ.

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