sive rats after 8 weeks of DOC-salt treatment (Table 1 and Fig. 3A).

This complex modulation of α-subunit mRNA isoforms in hypertension raises a question about the status of Na^+,K^+-ATPase β-subunit mRNA expression. Although all the biochemical properties of Na^+,K^+-ATPase are localized to the α subunit, the vectorial transport of ions is detected only in the intact αβ complex. We therefore assessed the amounts of β mRNA in the aortic and left ventricular RNA blots. We used the same preparation of mRNA and the same procedures as we used for α1 analysis. The β-subunit mRNA is modulated together with α1 mRNA. In the aorta, where a 2- to 3-fold increase in α1 mRNA and a 3- to 15-fold decrease in α2 mRNA are noted, a 2-fold increase in β mRNA (*16*) was detected (Fig. 1A). In skeletal muscle, where the major α isoform, α2, does not change and the minor α isoform, α1, increases twofold, an equivalent increase in β-subunit mRNA (*16*) was likewise detected under stringent hybridization conditions (Fig. 2). In the left ventricle, where the amount of α1 mRNA does not change, amounts of β mRNA likewise did not appear to change (*16*) (Fig. 3, B and C). Thus, the parallel modulation of β and α1 mRNAs suggests coordinate regulation of the β and α1 genes.

These results provide evidence that hypertension induces gene-specific modulation in vascular and cardiac tissues. Furthermore, the modulation of Na^+,K^+-ATPase is isoform-specific. The down regulation of the amounts of α2 mRNA (and probably also that of α3 in the left ventricle) in left ventricle and aorta of hypertensive rats may represent a deinduction of gene expression elicited in response to stimuli from increased intravascular pressure before development of hypertrophy. This deinduction appears to be closely related to pressure increments, since it was detected in left ventricular samples of hypertensive rats that were infused with A-II for 2 days (Table 1). The mechanisms of transduction of a mechanical pressor stimulus to a genetic response are unknown. Because of similar findings in aortas and skeletal muscle of hypertensive rats treated with DOC-salt (Figs. 1A and 2), the up regulation of α1- and β-subunit mRNA expression is clearly not pressure-related, but could be secondary to alterations in Na^+ flux induced by DOC-salt treatment; other studies have documented increased Na^+ influx in these tissues (*17, 18*). The increase in α1 mRNA correlates with increased vascular Na^+,K^+-ATPase activity in hypertensive rats treated with DOC-salt (*19, 20*).

The increase in α1 mRNA may be in response to an increase in Na^+ influx to maintain homeostatic levels of intracellular Na^+, and the decrease in the number of Na^+,K^+-ATPase mRNA molecules in the left ventricle might be an adaptive process to increments in vascular pressure. This decrease in Na^+,K^+-ATPase mRNA may underlie a decrease in the number of Na^+ pumps, which could be an endogenous equivalent of a ouabain-induced Na^+,K^+-ATPase inhibition resulting in cardiac inotropy. These changes in amounts of mRNA could represent transcriptional regulation, although changes in mRNA stability cannot be ruled out. Determination of the mechanisms by which specific Na^+,K^+-ATPase α- and β-subunit genes are modulated in hypertension may provide insight into the molecular mechanisms involved in the pathogenesis or consequences of hypertension.

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25 February 1988; accepted 9 May 1988

Amyloid β Protein Precursor Is Possibly a Heparan Sulfate Proteoglycan Core Protein

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The amyloid β protein peptide is a major constituent of amyloid plaque cores in Alzheimer's disease and is apparently derived from a higher molecular weight precursor. It is now shown that the core protein of a heparan sulfate proteoglycan secreted from a nerve cell line (PC12) has an amino acid sequence and a size very similar to those of the amyloid β protein precursor and that these molecules are antigenically related. This amyloid β protein precursor–related protein is not found in the conditioned medium of a variant cell line (F3 PC12) that does not secrete heparan sulfate proteoglycan. The synaptic localization and metabolism of this class of proteoglycans are consistent with its potential involvement in central nervous system dysfunction.

FILAMENTOUS AMYLOID STRUCTURES accumulate in the brain of patients with Alzheimer's disease (*1*), Down syndrome (*2*), and a number of infectious encephalopathies (*3*). In Alzheimer's disease, areas of disorganized neuropil called neuritic plaques surround a core of extracellular amyloid. The core has two major components, inorganic aluminosilicate (*4*) and a protein of approximately 4000 daltons termed β protein (*2*) or A4 (*5*). On the basis of a partial amino acid sequence of the β

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protein peptide, a cDNA clone was isolated from fetal human brain that encodes the precursor of β protein, which has a predicted molecular mass of about 79,000 daltons (*6*).

Heparan sulfate proteoglycans (HSPGs) are cell-surface and extracellular-matrix molecules that consist of a core protein to which are coupled heparan sulfate glycosaminoglycan (GAG) side chains (*7*). HSPGs are the major class of proteoglycans in the nervous system (*8*). Adhesion molecules such as fibronectin, laminin, and a putative nerve cell adhesion molecule (N-CAM) all interact strongly with heparin (*9*), as do molecules that are both growth factors and adhesion molecules, such as fibroblast growth factor and purpurin (*10*). Most of these adhesion-promoting molecules are associated with extracellular adhesion-mediating particles termed adherons (*11*). The cell-surface receptor for neuronal adherons is an HSPG (*12*).

As an initial step in determining the complete primary structure of an HSPG core protein, we purified the secreted form of a neuronal HSPG from PC12 cells and the amino acid sequence of the apparent amino-terminal peptide was obtained. Approximately 2 liters of serum-free PC12 (*13*) growth-conditioned medium from cells labeled with $^{35}SO_4$ were applied to a DE52 ion-exchange column. Elution with a NaCl gradient generated a single major peak of isotopically labeled HSPG. This peak was then chromatographed on a Sepharose CL4B column in 4*M* guanidine hydrochloride and eluted with an apparent molecular mass of about 200,000 daltons relative to protein molecular weight markers. Because many low molecular weight proteins have an affinity for HSPGs, guanidine hydrochloride was applied to the sizing column. In addition, to ensure that any amino acid sequence information obtained from this preparation was derived from the proteoglycan and not a contaminating protein, we metabolically labeled the proteoglycan with $^{35}SO_4$ before purification, digested it with trypsin, and chromatographed it on a reversed-phase high-performance liquid chromatography (HPLC) column (Fig. 1). Because only one proteoglycan has been detected in PC12 cells (*14*), all isotopically labeled tryptic peptides should be derived from this molecule. The isotope-containing peptides that were resolved were subjected to amino-terminal amino acid sequence analysis. The majority of the peptides in the tryptic digest were poorly resolved and contained several sequences, making the deduction of consensus sequences impossible. This observation is not surprising because proteoglycans are heavily glycosylated, which leads to extensive heterogeneity on reversed-phase columns. There was, however, one peak (Fig. 1, peptide 51) that contained a large amount of sulfate and was relatively uncontaminated by secondary peptides. Twenty-seven residues of this peak were sequenced (Fig. 2). A comparison of these sequences to known protein sequences showed a striking homology with the human amyloid β protein precursor (ABPP) (*6*). The sequence of peptide 51 begins at residue 18 of the predicted ABPP sequence and continues through residue 45 (*6*). The only difference in the sequence of peptide 51 is a substitution of arginine for lysine in position 40. If the initial 17 amino acids of the ABPP are a signal sequence (*6*), then residue 18 is probably the amino-terminal residue of the proteoglycan.

To confirm the relation between PC12 HSPG and the ABPP, we carried out electrophoresis of serum-free growth-conditioned medium on SDS-polyacrylamide gels. The proteins were transferred to nitro-

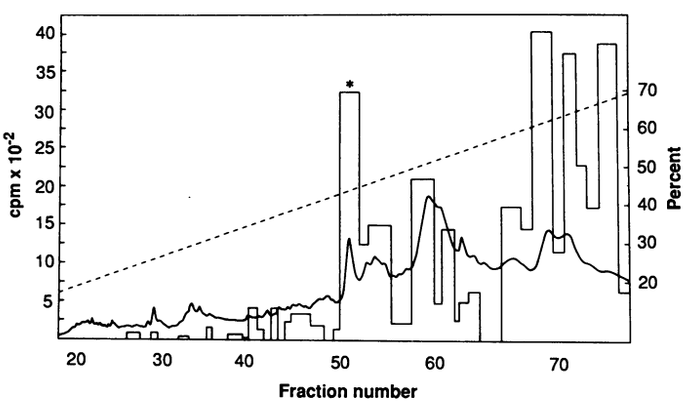

Fig. 1. Reversed-phase liquid chromatography of tryptic digest. Approximately 1 nmol of HSPG, which had been purified on DEAE and Sepharose 4B, was digested with trypsin (1 to 20 molar ratio with substrate) overnight at 37°C in 0.1*M* tris and 2*M* urea, *p*H 8.0, and pumped onto a 0.46 by 25 cm reversed-phase column (RP-300; Brownlee Labs) with a 0.1% trifluoroacetic acid:acetonitrile solvent system and eluted at 0.6 ml per minute with a 190-min gradient of 8 to 80% acetonitrile (- - -). Chromatography was at room temperature and the isotope in 5% of each fraction was determined by liquid scintillation spectrometry (bars). Optical density (OD_{214}) was also monitored (—). Full-scale OD_{214} was 0.1 and the OD_{214} of fraction 51 (*) was 0.022.

	20	25	30	35	40
Human ABPP	L E V P T D G N A G L L A E P Q I A M F C G	R	L N M H		
PC12 HSPG	L E V P T D G N A G L L A E P Q I A M F X G	K	X N M H		

Fig. 2. Data from the sequence analysis of a PC12 peptide (Fig. 1, fraction 51) were compared with that of human ABPP (*6*). Sequence homology between PC12 HSPG and human ABPP is identical, except for position 40 (boxed). The residue number from the amino terminus of human ABPP precursor is indicated at the top of the figure. Sequence analysis was done according to Esch (*24*). Leucine (151 pmol) was detected on the first cycle. On the second cycle there were 117 pmol of glutamic acid and 12 pmol of glycine, which suggests that the level of contaminating peptides was about 10%. The two Xs represent unresolved amino acids in the sequence. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; T, Thr; and V, Val.

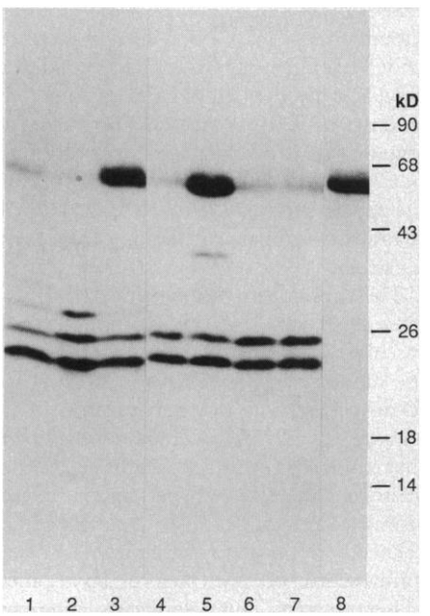

Fig. 3. Immunological detection of ABPP in growth-conditioned medium. Serum-free growth-conditioned medium from 5 × 10^6 cells was desalted on G25 columns in water, lyophilized, dissolved in an SDS sample buffer containing 5% β-mercaptoethanol, and electrophoresed on 14% polyacrylamide gels containing SDS (*25*). In some cases the secreted proteins were treated with heparinase (Sigma, 0.5 U/ml, 24 hours in 10 m*M* tris and 10^{-3}*M* $CaCl_2$, *p*H 7.0), or heparinase 2 (Sigma, 0.5 U/ml, 24 hours in 10 m*M* tris and 10^{-3} $CaCl_2$, *p*H 7.0). The proteins were transferred to nitrocellulose, reacted with a 1 to 1000 dilution of rabbit antibody to ABPP peptide (residues 556 to 566), or a monoclonal antibody (PG22) against the PC12 HSPG core protein (*14*). The immune complexes were detected with ^{125}I-labeled protein A (*26*). Lane 1, PC12; lane 2, PC12, heparinase; lane 3, PC12, heparinase 2; lane 4, PC12, grown in synthetic N_2 medium for 7 days; lane 5, PC12, N_2 medium, heparinase 2; lane 6, F3 variant; lane 7, F3 variant, heparinase 2; lane 8, PC12, heparinase 2, immunoblotted with PC12 antibody to HSPG core protein.

cellulose and reacted with a rabbit antiserum prepared against a synthetic peptide containing residues 556 to 566 of the ABPP (*6*, *15*) and with monoclonal antibody against the PC12 HSPG core protein (*14*). Because the ABPP appears to be a proteoglycan, we predicted that antisera prepared against synthetic peptides of the core protein would react more strongly with the antigen after the carbohydrate is removed with a heparan sulfate–degrading enzyme. In addition, because proteoglycans do not transfer to nitrocellulose well, immunoblot analysis may not detect the heavily glycosylated HSPG. Therefore, PC12 growth-conditioned medium was digested with heparinase and heparinase 2, which degrade heparin and heparin plus heparan sulfate, respectively (*16*). A secreted protein of 65,000 daltons was weakly detected by antibody to ABPP (Fig. 3, lane 1). The intensity of this reactivity was greatly increased by heparinase 2 digestion (lane 3) but not by heparinase (lane 2). These data suggest that heparinase is removing the glycosaminoglycan from the PC12 proteoglycan to generate a core protein that is recognized by the antiserum. Low amounts of unglycosylated core protein are secreted from the cells, but most of the protein is not detectable with the antisera before heparinase 2 treatment, either because the peptide epitopes are masked by carbohydrate or because the proteoglycan does not transfer well to nitrocellulose. To establish that the immunoreactive protein generated by heparinase 2 digestion of PC12 cell supernatant is the PC12 HSPG core protein, we carried out electrophoresis on duplicate samples on the same gel and analyzed both with antibody to ABPP and a monoclonal antibody against the HSPG (*14*). After heparinase 2 treatment, both antibodies detected proteins of identical mobilities (Fig. 3, lane 8); neither detected higher molecular mass proteins in the immunoblots before heparinase 2 digestion. These data show that ABPP and the PC12 HSPG core protein share the properties of size and sensitivity to heparan sulfate–degrading enzymes. They do not, however, formally eliminate the possibility that ABPP (possibly tyrosine-sulfated) is not the HSPG core protein but that it interacts strongly with HSPG during purification and in the gels.

To rule out the possibility that the antigen found in the conditioned medium of PC12 cells was adsorbed on the cell surface from the serum in which the cells were grown and then released into the serum-free medium, we carried out two experiments. Cells were grown in serum-free defined medium (*17*) for four cell divisions (1 week), washed with serum and protein-free medium, and serum-free growth-conditioned medium was prepared as before. When this medium was transferred to nitrocellulose and reacted with antibody to ABPP before and after heparinase 2 treatment, it reacted with the same intensities as cells grown in serum-containing medium (Fig. 3, lanes 4 and 5). To further test the possibility that the ABPP is an HSPG core protein, we reacted growth-conditioned medium from a variant (F3) of PC12 that contains no cell-surface HSPG (*18*) with the antibody to ABPP before and after heparinase 2 digestion. Although there was detectable ABPP antigen secreted by the F3 PC12 variant (Fig. 3, lane 6), the amount was not increased by heparinase 2 treatment (lane 7). This suggests that, while some core protein is secreted, the glycosylated HSPG is not present.

The 79,000-dalton (695-residue) ABPP has the structure of a cell-surface receptor; it contains a large extracellular domain with a putative membrane-spanning region near the carboxyl terminus (*6*). Heparan sulfate proteoglycans are also anchored in the plasma membrane by a terminal segment of their primary structure and are released (shed) into the extracellular space by proteolytic cleavage near the point of membrane insertion (*7*). The observations that the secreted form of the ABPP peptide is approximately 65,000 daltons (Fig. 3) and that the amino terminus is intact (Fig. 1) are consistent with a proteolytic cleavage site outside the membrane-spanning region at the carboxyl-terminal portion of the molecule that releases the proteoglycan from the cell surface. The predicted sequence of the ABPP is serine- and threonine-rich. There are five serine-glycine or glycine-serine dipeptides, which may be preferred sites for glycosaminoglycan chain attachment (*19*, *20*). There is a serine-glycine in the human ABPP 14 amino acids on the carboxyl-terminal side of the last sequenced methionine (Fig. 2, residue 43), which may account for the sulfate label in the peptide (*6*). There are 16 threonine residues followed by charged amino acids in the putative extracellular domain of the ABPP. These and the other threonine residues are also potential sites of GAG attachment. Although no complete sequence of an HSPG is known, the overall structure of the ABPP is similar to that of the class of proteoglycan core proteins (*7*, *20*).

Because HSPGs are found in synaptic vesicles and at synaptic contacts (*21*), an altered proteoglycan could lead to reductions in the area of the synapse or increases in the width of the synaptic cleft, and thus in a weakening of the synapse. The metabolism of HSPGs is also consistent with the involvement of their core proteins in neuritic plaque formation. Newly synthesized HSPG is incorporated into the cell surface and remains there or is released by limited proteolysis of the protein core from the membrane (*7*, *22*). Cell-surface HSPG is internalized and converted into a series of progressively smaller pieces by proteolysis of the core protein and endoglycosidic cleavage of the heparan sulfate side chains; the final degradation products are then released from the cell (*22*). Extracellular proteoglycans are internalized by cells and presumably degraded by a similar pathway. The proteolytic fragments of HSPG must reach a size of about 5000 daltons before they are totally deglycosylated (*22*). The deglycosylation of the small peptide could lead to its precipitation. The 42-residue amyloid peptide found in plaques may be the remnant of incompletely degraded HSPG core protein.

Note added in proof: The sequence of the protein shown in Fig. 2 is identical to that of the rat ABPP (*23*).

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15. Rabbit antiserum against synthetic peptide 556 to 566 of the predicted amyloid precursor sequence was provided by N. Dewji. The antiserum specifically stains a band of 65,000 daltons on immunoblots of hippocampus and also on immunoblots of neuroblastoma and teratocarcinoma cell lines that express mRNA for ABPP on RNA blots. The specific staining was eliminated by blocking with synthetic peptide. The major 65,000-dalton band on SDS-polyacrylamide gels also reacted with antiserum to four other synthetic peptides from different regions

of the amyloid precursor protein. The reactivity of the peptides to this protein was increased by β-elimination or treatment with heparinase 2. In addition to the 65,000-dalton protein, some of these antisera also react with a doublet of 105,000 and 110,000 daltons in brain and PC12 supernatant. Because these proteins can be degraded with heparinase 2 to a 65,000-dalton protein, it is possible that they are partially glycosylated forms of the same core protein.
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4 February 1988; accepted 23 May 1988

Yeast *KEX2* Endopeptidase Correctly Cleaves a Neuroendocrine Prohormone in Mammalian Cells

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Mammalian cell lines (BSC-40, NG108-15, and GH_4C_1) that cannot process the murine neuroendocrine peptide precursor prepro-opiomelanocortin (mPOMC) when its synthesis is directed by a vaccinia virus vector were coinfected with a second recombinant vaccinia virus carrying the yeast *KEX2* gene, which encodes an endopeptidase that cleaves at pairs of basic amino acid residues. mPOMC was cleaved intracellularly to a set of product peptides normally found in vivo, including mature γ-lipotropin and β-endorphin$_{1-31}$. In GH_4C_1 cells (a rat pituitary line), product peptides were incorporated into stored secretory granules. These results suggest that the inability of any particular cell line to process a prohormone precursor is due to the absence of a suitable endogenous processing enzyme.

PREPRO-OPIOMELANOCORTIN (POMC) is one of the most thoroughly characterized polyprotein precursors (*1*). The processing of POMC is complex and varies in different cell types (*2*). Each cryptic peptide in POMC is flanked by a pair of basic amino acid residues, namely, -Lys-Arg-, -Arg-Arg-, -Arg-Lys-, -Lys-Lys- (Fig. 1), a motif found in essentially all prohormones (*1*). Liberation of the bioactive segments is a two-step process (*3*): (i) the precursor is cleaved at the carboxyl side of the pair of basic residues by a trypsin-like endopeptidase; and (ii) the doublet of basic residues exposed on the carboxyl-terminal end is removed by an enzyme similar to carboxypeptidase B.

The biochemical basis for the tissue specificity of processing reactions is not understood. Several factors may be involved, including: (i) selective expression of distinct processing enzymes, (ii) differential compartmentation of either one or more proteases or the precursor, and (iii) modulation of cleavage-site accessibility by differential modification of the precursor. In order to distinguish among these possibilities, identification of one or more endopeptidases responsible for the initial cleavages in prohormone processing is essential. Several classes of proteases (*3–9*) have been proposed as candidates for the true prohormone endoprotease. A physiologically relevant role has not been established for any of these enzymes in vivo, however. In contrast, the enzyme required for excision of a yeast peptide hormone (α-factor mating pheromone) from its precursor has been unequivocally identified [for review, see (*10*)]. The *KEX2* gene of *Saccharomyces cerevisiae* (baker's yeast) encodes a membrane-bound endopeptidase specific for cleaving on the carboxyl side of pairs of basic residues that contain arginine (-Lys-Arg- and -Arg-Arg-) (*11*). The *KEX2* enzyme is an unusual serine protease (*12*) that is strictly Ca^{2+}-dependent and has a neutral *p*H optimum (*11–13*).

We describe here the identification of cell lines and the establishment of conditions that permitted the detection of proper processing in vivo of a mammalian prohormone by the precursor-cleaving enzyme encoded by the yeast *KEX2* gene.

Cell lines were infected with recombinant vaccinia viruses expressing the cDNAs for prepro-enkephalin (*14*) or mouse POMC (mPOMC) (*15*). The fate of these precursors was observed by radiolabeling and electrophoretic, chromatographic, and immunological analysis. Three cell lines (NG108-15, GH_4C_1, and BSC-40 cells) (*16*) were unable to release bioactive peptides from these precursors (*14, 15a*), (Fig. 2).

To determine whether the *KEX2* gene could produce a functional membrane-bound enzyme in tissue culture cells, we inserted the *KEX2* gene into a vaccinia virus expression vector by the marker transfer method (*14, 17*). When a variety of mammalian cell lines were infected with this construction (VV:*KEX2*), a large increase in an activity capable of cleaving a specific fluorogenic peptide substrate was observed (Table 1). Depending on the cell type, the specific activity in cells infected with VV:*KEX2* was 16 to 33 times as great as that in mock-infected cells or cells infected by control viruses (VV:WT and VV:mPOMC) (Table

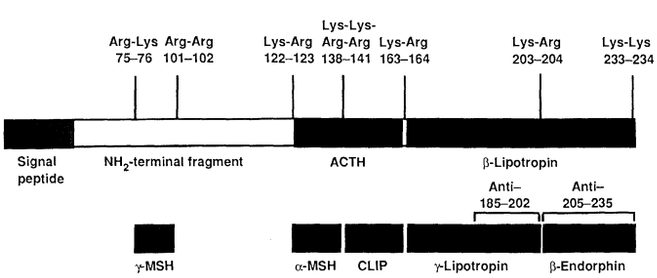

Fig. 1. Structure and processing of the mouse polyprotein precursor, POMC. Mouse POMC (mPOMC) is a 235-residue polypeptide that is processed by cleavage at the indicated pairs of basic amino acid residues into distinct sets of peptide hormones in the anterior and neurointermediate lobes of the pituitary (*1, 2*). Numbering of amino acids in mPOMC is according to that predicted from the cDNA sequence (*30*), beginning with the initiator methionine of the signal peptide. The two specific antisera used in this study are directed against the regions indicated by the brackets. Antiserum 185–202 is selective for γ-LPH (but will also recognize β-LPH and intact mPOMC); similarly, antiserum 205–235 is selective for β-END (but will also recognize β-LPH and intact mPOMC). ACTH, adrenocorticotropic hormone; γ-MSH, γ-melanocyte–stimulating hormone; α-MSH, α-melanocyte–stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide.

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