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A 16 kDa protein co-isolating with gap junctions from brain tissue belonging to the class of proteolipids of the vacuolar H^+-ATPases

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A 16 kDa protein from an enriched gap junction preparation was isolated from bovine brain tissues. N-terminal amino acid microsequencing of the first 20 amino acids showed a complete homology with a recently published sequence of a proteolipid from a vacuolar H^+-ATPase from chromaffin granules. Incubation of the brain gap junction preparation with ^{14}C-*N,N'*-dicyclohexylcarbodiimide showed a significant binding of this compound to the 16 kDa protein, indicating that a proton binding site also occurs within that particular protein. The data suggest that this 16 kDa protein, which has also been described in gap junction preparations from various other tissues, belongs to the proton transporting ATPase.

Gap junction; Amino acid sequence; Sequence homology; ATPase, H^+-; (Bovine brain)

1. INTRODUCTION

Gap junctions are considered to play a crucial role in the conduction of signals from cell-to-cell. Signal transfer is maintained by channel-forming proteins which have been isolated and characterized by microsequencing and/or cDNA cloning [1–4]. The general concept derived from these findings is that gap junctions consist of a family of diverse but homologous proteins, the pattern of which varies from tissue to tissue. These proteins, here referred to as connexins (cx) first suggested by Goodenough [3] vary in molecular masses between 26 kDa (cx26) [5], 32 kDa (cx32) [1,2] and 43 kDa (cx43) [3] based on their predicted amino acid sequence from their encoding cDNAs. A 16 kDa protein frequently reported in diverse tissues [6] is thought to represent a gap junction constituent. However, the nature of this protein remains obscure since homology based on immunochemical criteria and two-dimensional peptide mapping [7] on connexins have not been evidenced. By a novel technique for the isolation of gap junctions from brain tissues reasonably pure preparations of gap junction-like structures from bovine brain as identified by negative staining electron microscopy were obtained. The predominant component of this preparation is a 16 kDa protein. It was electro-eluted from stained gels and the first 20 residues were micro-sequenced by automated Edman degradation; 19 out of 20 amino acids from the N-terminal of this polypeptide showed a totally coincident homology with the sequence of a recently isolated and sequenced proteolipid of a vacuolar H^+-ATPase [8].

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2. MATERIALS AND METHODS

2.1. *Isolation of bovine brain gap junctions*

Fresh bovine brain tissue, 0.5–1 h after slaughter, with its meninges stripped off, was homogenized with an ultra-turrax until no lumps were discernible. The isolation procedures employed a combination of two methods, entailing the separation of myelin [9], and the isolation of gap junctions from the crude plasma membrane fraction according to [10].

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2.2. *SDS-PAGE and electro-elution*

SDS-PAGE and electro-elution of the gap junction plaques were conducted as described [11]. Electro-elution of Coomassie stained gel strips of the 16 kDa band were carried out by adopting the method as described [12]. A few precautions were taken to minimise amino-terminal blockage of the proteins, such as using ammonium bicarbonate buffer instead of glycine and phosphate buffer, no urea was used.

2.3. *N-terminal amino acid sequencing*

The electro-eluted protein was lyophilized and submitted to sequence analysis in an Applied Biosystems 470 A pulsed liquid protein sequencer.

2.4. *DCCD labelling*

200 μl of a ^{14}C-DCCD stock solution (spec. act. 50 μCi/μmol) was evacuated in a Speedvac until the liquid was evaporated. The sample was then redissolved in 100 μl of absolute ethanol to a final activity of 0.1 μCi/μl.

30 μg of the purified 16 kDa protein as well as of the bovine brain plasma membrane were mixed with 20 μl of the redissolved ^{14}C-DCCD solution, to provide a final activity of 0.05 μCi/μg protein for both samples, respectively.

After 24 h of incubation at 4°C both samples were centrifuged in an Eppendorf-Microfuge for 10 min. The samples were processed by electrophoresis [11] and subjected to autoradiography.

3. RESULTS AND DISCUSSION

The gap junctions obtained by the above described technique consist of about 50–80% of membranous plaques revealing gap junction-like structures by negative staining and subsequent electron microscopy. The remaining fraction consists of split gap junctions, unidentifiable smooth-surfaced vacuoles and some flocculent material (fig.1). The gap junction plaques showed the typical paracrystalline pattern often arranged in a chess-board like fashion. High resolution electron microscopy (EM) shows connexon-like particles with central deposits of negative stain within them indicative of the existence of central pores (not shown). It is evident from the electron micrographs that these plaques constitute the main membranous fraction of the preparation.

SDS-PAGE shows a dominant band at the position of 16 kDa with some other bands at 34 kDa and between 43 kDa and 55 kDa (inset fig.1). Densitometric tracing of Coomassie blue stained gels indicate that the 16 kDa band represents about 60% of the complete preparation.

Electro-elution of the 16 kDa band from stained gels and subsequent N-terminal amino acid microsequencing of the first 20 amino acids yielded a polypeptide which is identical with part of the sequence, recently reported for the proteolipid of a vacuolar H^+-ATPase [8]. The sequenced part of our polypeptide coincides with positions 7–26 of the ATPase polypeptide (table 1). As the initiating methionine was not found, it is quite likely that our protein is an internal fragment of the original ATPase proteolipid having lost residues 1–6.

From a significant sequence homology between the 16 kDa vacuolar ATPase proteolipid from bovine chromaffin granules and the 8 kDa proteolipids of electron transport ATPases, e.g. the chloroplast ATP synthase proteolipid CF_0III, a relation of these polypeptides has been deduced [8]. In table 1 we tried to maximize this homology in the alignment by introducing gaps. However, the similarity between the polypeptides shown is rather weak and in addition we could not detect an immunochemical crossreaction of antisera against CF_0III from spinach in Western blots with the gap junction preparation (data not shown). An antiserum against the 8 kDa proteolipid ATPase from bovine mitochondria, or from yeast mitochondria, was not available to us.

Since proton-transporting ATPases are known to contain a hydrophobic membrane sector with three or more membrane spanning polypeptides, one of which is an *N,N'*-dicyclohexylcarbodiimide (DCCD) binding protein [13,14] being involved in the proton conduction, we have labelled our plaque preparation with ^{14}C-DCCD, SDS-PAGE of the labelled plaque preparation and subsequent autoradiography showed an intense labelling at the 16 kDa position, with further labelling at 8 kDa (fig.2). A 16 kDa signal was also found in membrane preparations from bovine brain indicating that a DCCD binding 16 kDa protein is present in brain plasma membranes.

Although increasing evidence indicates that different proteins contribute to the formation of gap junctions, the exact composition of its channel-forming constituents is still under debate. Immunofluorescence and electron microscopical immunocytochemistry provided evidence that at least two connexins (cx26 and cx32) co-localize within the same plaque in liver gap junctions [17,18]. A recently cloned and completely sequenced gap junction protein from heart tissue (cx43) [3] has also been shown to participate in gap junction complements of various organs [19]. Though cur-

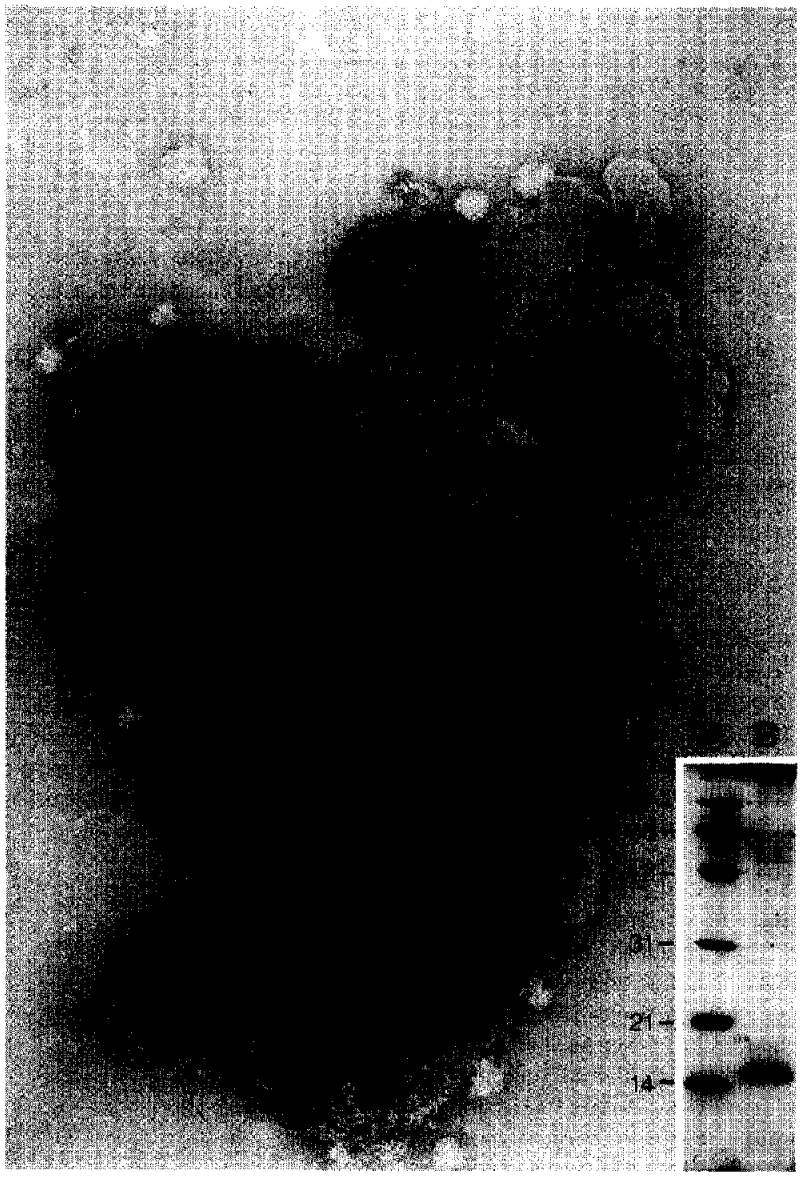

Fig.1. Electron micrograph of a negatively stained preparation of isolated bovine brain gap junctions after myelin separation displaying the substructure typical of gap junctions. (Inset) A SDS-PAGE electropherogram: lane A showing the low molecular mass standards; lane B, the isolated bovine gap junction.

rent models on the molecular composition of gap junction channels purport a hexameric orientation of the connexins, direct evidence as to whether this set of molecules consists of hetero- or homomeric proteins is as yet not forthcoming. Moreover, there is no consensus regarding the connexin family constituting the sole gap junction-forming proteins. The lens fiber protein (MIP 26), for which the entire sequence has been determined [20], has no homology with the connexin proteins.

In addition, a 16 kDa protein isolated from different species and phyla has frequently been propounded as the major structural component of gap junctions [6,7].

Table 1

The N-terminal amino acid sequence of the 16 kDa polypeptide from bovine brain gap junctions, aligned with the sequence of a 16 kDa proteolipid from the vacuolar H^+-ATPase of bovine chromaffin granules, a homologous reading frame from yeast, and with homologous 8 kDa proteolipids of electron transport ATPases

	5 15	
gap junct.	xPEYASFFAVMGASAAMVFSxxxxx	
chrom. gr.	MSEAKNGPEYASFFAVMGASAAMVFSAL..GAAYGTA.KSGTGIAAMSVMR.PEMIMK.SIIP	etc.
yeast vac.	MTEL..CPVYAPFFGAIGCASAIIFTSL..GAAYGTA.KSGVGICATCVLR.PDLLFK.NIVP	etc.
bov. F_o9	DI...DTAAKFIGA.GAATVGVA...GSGAGIGTVFGSLI.I...GYARNPSL..KQQLFS	etc.
yeast F_o9	MQL...VLAAKYIGA.GISTIGLL...GAGIGIAIVFAALI....NGVSRNPSI..KDTVFP	etc.
CF_oIII	MNPL...IAAASVIAA.GLA.VGLAS.IGPGVGQGTAAGQAV....EGIARQPEAEGK..IRG	etc.

gap junct., 16 kDa polypeptide from bovine brain gap junction preparations; chrom.gr., 16 kDa proteolipid from bovine vacuolar H^+-ATPase from chromaffin granules [8]; yeast vac., homologous sequence deduced from a reading frame from *Saccharomyces cerevisiae* [15]; bov. F_o9, 8 kDa proteolipid from bovine mitochondria ATPase [16]; yeast F_o9, 8 kDa proteolipid from yeast mitochondria ATPase [16]; CF_oIII, 8 kDa proteolipid from spinach chloroplast ATP synthase [16]

By the detergent treatment with 1% Triton X-100 on complete tissue homogenates instead of on isolated plasma membrane fractions, a 16 kDa protein was consistently obtained [6]. This bore no relationship to the connexin family from biochemical and immunochemical results. Although we used a different approach in isolating the 16 kDa protein from brain, two main reasons point to the identical nature of this protein to that described earlier [6]. First, from SDS-PAGE analysis it follows that in both procedures a 16 kDa polypeptide co-isolates with the plasma membrane fraction consisting of gap junction-like plaques under EM. Secondly, a comparison of the amino acid sequence with an unpublished sequence of a 16 kDa protein from liver isolated according to the Finbow technique [6] (Hertzberg, personal communication) revealed a high degree of homology differing only in positions 5 and 15 to our sequence.

The high incidence in homology with the vacuolar proteolipid suggests for the first time that the 16 kDa protein co-isolating with gap junctions belongs to the class of vacuolar H^+-ATPase. Further the ^{14}C-DCCD binding to the 16 kDa protein, isolated by us from bovine brain, reinforces the notion that this protein exhibits a proton binding site; as shown for the proteolipid of the H^+-ATPase, isolated from chromaffin granules [8]. The possibility of the 16 kDa polypeptide isolated from preparations of bovine chromaffin granules [8], being a 16 kDa gap junction protein, can be ruled out: from the unicellular yeast, *Saccharomyces cerevisiae*, which does not display gap junctions, a gene was isolated and sequenced, encoding a 16 kDa proteolipid with high sequence

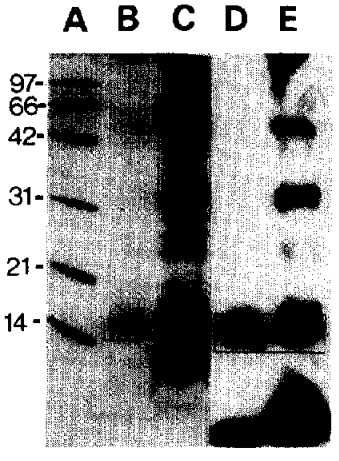

Fig.2. SDS-PAGE electropherogram. Lanes: A, low molecular mass standards; B, bovine brain gap junctions exposed for autoradiography; C, bovine brain plasma membrane exposed for autoradiography; D, ^{14}C-DCCD-labelled bovine brain gap junction preparation corresponding to lane B; E, ^{14}C-DCCD labelled bovine brain plasma membrane preparation corresponding to lane C. For clarity, the 16 kDa band in lanes B, D and E has been boxed.

homology to the 16 kDa polypeptide from bovine chromaffin granules [15], cf. table 1.

The crucial issue that still remains to be addressed is whether the 16 kDa protein is indeed a part of the gap junction protein complex.

Until now EM has only provided indirect evidence based on the abundance of gap junction-like assemblies in the preparations. Since some contaminants appeared in the form of smooth surfaced vacuoles and flocculent material in the preparation, it is conceivable that some of this material may constitute the 16 kDa protein. However, the quantitative evaluation of the electron micrographs in comparison to SDS-PAGE electropherogram, where a predominance of the 16 kDa band persisted, speaks against such an assumption. An alternative interpretation [21] that the gap junction-like assemblies present in enriched 16 kDa protein preparations originating from intracytoplasmic membranes with integral membrane proteins aligned in a paracrystalline pattern mimicking gap junction plaques cannot be ruled out; since ATPases are known to assemble in geometrical patterns under isolation conditions [22].

To determine unequivocally whether the 16 kDa vacuolar ATPase proteolipid co-isolating with gap junctions, is part of the junctional domain, more morphological and functional criteria such as in situ EM immunocytochemical labelling with antibodies to this protein need to be satisfied.

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