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cAMP-dependent protein kinase phosphorylates gap junction protein in lens cortex but not in lens nucleus

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MP70 (a 70 kDa membrane protein) is a component of the gap junctions of the young fibre cells in the lens outer cortex. In the older fibres deeper in the mammalian lens (lens nucleus), MP70 is processed to MP38 by cleavage and removal of the carboxy terminal half. It is shown here that cortical MP70, and its derivative MP64, can be phosphorylated with cAMP-dependent protein kinase. In contrast, MP38 from the lens nucleus is not phosphorylated by the enzyme. Proteolytic processing and this lens region specific phosphorylation are relevant for the future development of functional assays for lens gap junctions.

Introduction

Gap junctions provide pathways for intercellular communication in the lens and thus play an important role for tissue homeostasis and lens transparency. A 70 kDa membrane protein (MP70) localized in the lens fibre gap junctions, is the largest member of the divergent connexin family of proteins identified so far [1]. In isolated junctions, MP70 is always present along with MP64, which has been shown to be derived from MP70, most probably by cleavage [1,2]. Detergent solubilized MP70/64 has an appearance consistent with connexon pairs and most probably forms the transmembrane channels in intact gap junctions [3]. While connexins from other tissues have channel properties and are regulated by phosphorylation [4], such data is not yet available for MP70/64. We now report that MP70/64 from the younger fibre cells in the lens cortex can be phosphorylated in a cAMP-dependent way. In contrast, the processed form of this junction polypeptide in the lens nucleus, MP38 [2], has lost this capability.

Materials and Methods

Lenses from sheep generally less than 1 year old were extracted from eyes within minutes of death and collected on ice. Lenses were frozen at −90 °C 2–3 hours after extraction and stored at the same temperature.

Phosphorylation of lens junction protein with endogenous cAMP-dependent protein kinase was studied using homogenates from separate lens regions. Lenses were decapsulated and dissected into outer cortex, inner cortex and nucleus according to Kistler and Bullivant [2]. Tissue was homogenized in 1 mM Tris-HCl (pH 8), 5 mM EGTA, and protein concentration determined by absorption at 280 nm. Aliquots of outer cortex and nucleus homogenates containing 60 mg protein were adjusted to 300 μl with homogenization buffer. After further addition of 300 μl 20 mM imidazole (pH 7.4), 1 mM $MgCl_2$ and 6 μl 2 mM cAMP, the mixtures were preincubated for 5 min at 37 °C. Controls contained 0.7 mg protein kinase inhibitor isolated from bovine heart [5]. 6 μl [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml; Amersham, Buckinghamshire, U.K.) and 6 μl 5 mM ATP were added, and the reaction mixtures incubated for 10 min at 37 °C. Reactions were terminated by adding 100 μl 100 mM EDTA. Membranes were purified by urea/alkali stripping, and membrane proteins analysed by SDS-PAGE (10% acrylamide) and autoradiography according to Kistler and Bullivant [2]. All reagents were from Sigma Chemical Co., St. Louis, U.S.A., unless specified otherwise.

Phosphorylated junction protein was identified by immunoprecipitation with monoclonal anti-MP70 antibodies [6]. For this, phosphorylated and urea/alkali stripped cortical membranes from two aliquots were pooled, and gap junctions dissociated with 0.2% Non-

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idet NP-40. Detergent resistant membranes were pelleted, and soluble MP70/64 was precipitated from the supernatants with anti-MP70 (IgM) and anti-IgM conjugated agarose beads. For controls, monoclonal antibodies (IgM) against a non-related 17 kDa intrinsic membrane protein [7] were used instead of anti-MP70.

Phosphorylation of MP70/64 and its cleaved forms was studied using purified lens fibre membranes and commercial cAMP-dependent protein kinase from rabbit muscle (Sigma). For this, urea/alkali stripped membranes (400 μg protein) from outer cortex or nucleus were pelleted and resuspended in 400 μl 20 mM imidazole (pH 7.4), 1 mM $MgCl_2$, 4 μl 2 mM cAMP and 4 μl 10 mg/ml protein kinase were added and the mixtures preincubated at 37°C for 10 min. After the addition of 2 μl [γ-^{32}P]ATP and 4 μl 5 mM ATP, the samples were incubated for 10 min at 37°C. Reactions were stopped with 200 μl 100 mM EDTA and membranes washed with phosphate-buffered saline (PBS). To produce the amino terminal MP30 fragment of MP70/64 (Christie, D., personal communication), membranes were resuspended in 500 μl 10 μg/ml trypsin in PBS and incubated for 40 min at 37°C. Proteolysis was stopped by the addition of 100 μl 1/100 diluted diisopropyl fluorophosphate.

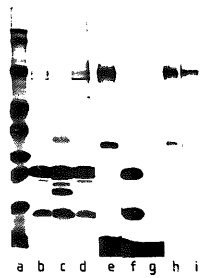

Fig. 1. Protein phosphorylation in homogenates of different lens regions and isolation of membranes. Lanes a–d: stained gels, lanes e–j: autoradiographs. (a) Molecular weight markers from top, 10^{-3} M_r: 205, myosin; 116, β-galactosidase; 97, phosphorylase *b*; 66, bovine serum albumin; 45, ovalbumin; 36, glyceraldehyde-3-phosphate dehydrogenase; 29, carbonic anhydrase; 24, trypsinogen; 20, soybean trypsin inhibitor; 14, α-lactalbumin. (b,e,h) Membranes from outer cortex. (c,f) Membranes from lens nucleus. (d,g) Control with Walsh inhibitor, membranes from outer cortex. (i,j) Immunoprecipitation of junctional protein. (i) Precipitation with anti-MP70. (j) Control using anti-MP17.

Results

Phosphorylation of lens proteins with the lens endogenous cAMP-dependent protein kinase was carried out separately in homogenates of outer cortex and of lens nucleus. Membranes were subsequently purified by urea/alkali stripping. The dissection of the younger cortical fibre cells from the older cells in the lens nucleus was effective to the point that the Coomassie-stained gel of outer cortex membranes revealed the junctional component MP70/64, but did not show detectable amounts of the processed form MP38 (Fig. 1, lanes b and d). Apparently, all MP70/64 was processed to MP38 in membranes from the lens nucleus (Fig. 1, lane c). In the corresponding autoradiograph (Fig. 1, lanes e–g), MP64 was the most strongly labeled polypeptide in outer cortex membranes (Fig. 1, lane e). MP70 was not significantly labeled and, although it seems unlikely, we cannot exclude the possibility that MP64 is the phosphorylated product of MP70 under these conditions. Another phosphorylated polypeptide in the vicinity of M_r 38000 on the gel, has apparently no Coomassie-stained counterpart (Fig. 1, cf lane b and e). This band may represent an unidentified minor lens protein or, less likely, minor amounts of phosphorylated MP38 in the lens outer cortex. MIP26 and MP17, the two most prominent lens membrane proteins, and not related to MP70, were not phosphorylated in lens outer cortex homogenates. MP38 in the lens nucleus was not phosphorylated (Fig. 1, lane f). Labeling of MIP26 and MP17 showed that lens protein kinase was active in the homogenate. Hence, cleavage of MP70/64 to MP38 or age-related modification of MP38 in the lens nucleus abolishes the phosphorylation capability of this polypeptide. Membrane proteins were generally not labeled in the presence of Walsh inhibitor, indicating the cAMP-dependency of these phosphorylations (Fig. 1, lane g).

Phosphorylated MP64 in outer cortex membranes (Fig. 1, lane h) was identified by solubilization with detergent and precipitation with anti-MP70 antibodies (Fig. 1, lane i). Anti-MP17 antibodies did not precipitate any radioactive material from this outer cortex preparation (Fig. 1, lane j).

The selective phosphorylation of cortical gap junction polypeptide but not of the cleaved product in the lens nucleus, was further examined using purified membranes and cAMP-dependent protein kinase from rabbit muscle. Under these conditions, MP70 and MP64 appeared strongly labeled, along with many other lens membrane proteins (Fig. 2, lanes b and e). Tryptic digestion of this labeled preparation removed most of the radioactivity from the membranes (Fig. 2, lanes c and f). Cleavage of MP70/64 with trypsin produced the membrane-bound, amino-terminal peptide MP30 (Christie, D., personal communication), which is clearly visible on the Coomassie-stained gel, but not on the autoradiograph (Fig. 2, lanes c and f). This suggests that phosphorylation of MP70/64 occurs in the carboxy-

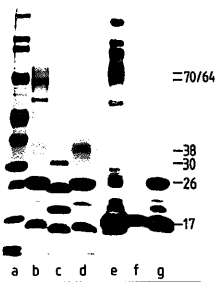

Fig. 2. Phosphorylation of isolated membranes with cAMP-dependent protein kinase from rabbit muscle. Lanes a–d: stained gels, lanes e–g: autoradiographs. (a) Molecular weight markers. (b,e) Outer cortex membranes. (c,f) Cortical membranes trypsinized after phosphorylation. (d,g) Membranes from lens nucleus.

terminal half of the molecule. This is further supported by the finding that MP38 in membranes isolated from the lens nucleus was not phosphorylated by the exogenous protein kinase (Fig. 2, lanes d and g).

Discussion

Phosphorylation of the major lens membrane proteins MIP26 and MP17 has previously been examined [7,8] and regional differences in the phosphorylation of MIP26 have most recently been demonstrated [9]. Our results are consistent with these reports, and show for the first time that the lens gap junction component MP70/64 is phosphorylated in vitro with cAMP-dependent protein kinase. Phosphorylation with cAMP-dependent protein kinase has previously been demonstrated for another gap junction polypeptide, the 27 kDa gap junction polypeptide from liver [10], and had an enhancing effect on junctional conductance between hepatocytes [11]. Such a structure-function relationship has yet to be demonstrated for lens MP70/64. The development of a functional assay for lens gap junction components will have to take into account that the lens tissue is non-uniform. Age-related modification or proteolytic processing apparently affects phosphorylation, which may have a regulatory function. Gap junction conductance or its regulation probably differs between the outer cortex and the lens nucleus. Hence, the interpretation of physiological experiments with isolated or reconstituted membranes will have to consider the relative presence of MP70, MP64 and MP38, and whether or not this junctional protein is phosphorylated.

Acknowledgements

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