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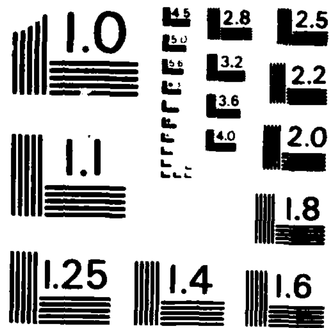

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A bacteriophage (λgt11) cDNA expression library, derived from bovine brain mRNA, was screened with polyclonal antibodies raised against the plasma membrane calcium pumping ATPase (Ca^{2+}-ATPase). A positive phage was isolated and purified to homogeneity in three subsequent rescreenings. The expressed protein product was shown to be reactive with antibodies against the calmodulin binding domain of the Ca^{2+}-ATPase. The cDNA sequence showed homlogy with the SR Ca^{2+}-ATPase 3'-end noncoding region and the

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derived protein sequence bore homology with the protein sequences of myosin light chain kinase calmodulin binding domain from smooth and skeletal muscle.

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CLONING OF A cDNA ENCODING THE CALMODULIN BINDING DOMAIN OF THE PLASMA MEMBRANE Ca^{2+}-ATPase

Final Report

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Nov. 17, 1986

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FINAL REPORT OF RESEARCH FINDINGS

Introduction

The divalent cation, Ca^{2+}, acts as a major second messenger for numerous processes in biological systems. Studies over the past 15 years have provided a substantial understanding of the molecular bases through which calcium signals act via intracellular receptors such as calmodulin (CaM) as has been summarized in numerous recent reviews (see for example, Klee and Vanaman, 1982; Veigl *et al.*, 1984). However, the ability of the cell to use free Ca^{2+} concentration as a regulatory signal results from the fact that resting state concentrations of this cation are normally maintained at below micromolar concentrations by the action of a set of sequestration systems found in all cell types. On stimulation, cytosolic Ca^{2+} levels rise transiently via influx and release from internal stores to concentrations of 10^{-5} to 10^{-4} M to give increases in the activities of those enzymes involved in mounting the required tissue specific response. Termination of this response occurs when Ca^{2+}-levels are returned to the resting state levels. While bulk transport of Ca^{2+} is accomplished by a Ca^{2+}-Na^{+} exchange protein, this system only brings the intracellular Ca^{2+} levels to about 10^{-5} M. The plasma membrane Ca^{2+} pumping ATPase (Ca^{2+}-ATPase) is then utilized to reach the resting level.

The major form of the Ca^{2+}-ATPase found in all cell types is that found in the plasma membrane. To date, only the human erythrocyte membrane enzyme has been studied in detail. As set forth in the following sections, this enzyme is activated by CaM linking its activity to cellular responses. Substantial work is currently in progress in this and other laboratories to elucidate the structural features of this enzyme which specify membrane association, ATP hydrolysis, cation translocation and CaM regulation. Traditional techniques of protein chemistry applied to the purified ATPase have been the only approach to such studies to date. The large size of this enzyme (138 kDa) makes such studies difficult and precludes determination of the entire structure of the enzyme. We have recently developed a series of immunoreagents which are suitable for use in attempts to clone the ATPase. This would greatly facilitate structural studies as well as provide the necessary probes for examining its structural gene, its expression in different cell types, its distribution throughout animal species and possible alteration of its structure or expression in various disease states.

Final Report

We have successfully cloned a portion of the Ca^{2+}-ATPase of plasma membranes. This was done by screening a cDNA library inserted into a bacteriophage vector (λgt11) that produces a fusion protein of β-galactosidase and the inserted cDNA. The screening was done with a polyclonal antibody that had been raised in rabbits against human erythrocyte Ca^{2+}-ATPase and was affinity purified by passing whole immune serum over over a column containing covalently bound Ca^{2+}-ATPase. The Ca^{2+}-ATPase specfic antibody was then eluted with a low pH buffer. The λgt11 cDNA library was made from bovine brain messenger RNA by standard cDNA synthesis techniques.

As noted, the cloning vector used in these experiments yields an expressed protein that is coded for by the inserted cDNA. This allowed us to test the validity of a putive positive bacteriophage in a number of ways.

First, a cellular extract made from bacteria infected with the bacteriophage believed to contain a cDNA insert coding for a portion of the Ca^{2+}-ATPase was resolved by denaturing polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The expressed protein was detected by Ca^{2+}-ATPase specific immunostaining and was shown to be about 130kDa in size. This is to be expected since the fusion protein will be the sum of the molecular weights of the β-galactosidase and the Ca^{2+}-ATPase fragment coded for by the cDNA. This piece of data shows that the isolated cDNA codes for a 16 kDa piece of the Ca^{2+}-ATPase, or about 20% of the intact protein. Additional evidence shows that antibodies purified against the fusion protein react with purified Ca^{2+}-ATPase. In particular, these antibodies react only with fragments generated by a limited tryptic digest of the Ca^{2+}-ATPase known to contain the calmodulin binding domain (Zurini, et al., 1984). When these antibodies are added to a Ca^{2+}-ATPase assay they give partial stimulation of the Ca^{2+}-ATPase but not complete stimulation to the level obtained when calmodulin is used. These antibodies also block further activation by calmodulin to the Ca^{2+}-ATPase's normal level of stimulation. These data indicate that we have successfully cloned a cDNA coding for the calmodulin binding domain of the plasma membrane Ca^{2+}-ATPase.

Using the Sanger dideoxy sequencing method we have sequenced the entire cDNA. We have found that the cDNA is 1456 bp long and codes for 93 amino acids. There are 1177 bp of 3'-end noncoding region. Protein sequence homology comparisons with the calmodulin binding domains of skeletal muscle myosin light chain kinase (MLCK) (Blumenthal, et al., 1985) and smooth muscle MLCK (A. Means, personal communication) show regions of strong homology. These regions are conserved in both MLCK sequences, again suggesting we have isolated a cDNA encoding the calmodulin binding domain of the Ca^{2+}-ATPase. Comparison at the DNA level with the 3'-end of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase shows homology. Interestingly, this homology follows into the coding sequence of the plasma membrane (PM) Ca^{2+}-ATPase, but falls off when the sequence coding for the calmodulin binding domain is encountered. The SR Ca^{2+}-ATPase is not stimulated by calmodulin.

We have, therefore, isolated a cDNA fragment coding for a portion of the Ca^{2+}-ATPase from bovine brain. The validity of this fragment coding for Ca^{2+}-ATPase has been confirmed by several tests based on reactivity of a protein coded for by the cDNA with antibodies against purifed Ca^{2+}-ATPase. Also, the protein sequence deduced from cDNA sequence is homologous to other calmodulin binding domains isolated from other calmodulin stimulated enzymes. The cDNA sequence is also homologous to the SR Ca^{2+}-ATPase cDNA sequence.

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Scientific Personnel Supported by DAAG29-83-G-0006

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