Molecular Characterization of Rat Cardiac Sarcolemmal Ca^{2+}/Mg^{2+} Ecto-ATPase

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by

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Molecular Characterization of Rat Cardiac Sarcolemmal Ca^{2+}/Mg^{2+} Ecto-ATPase

by

SUBBURAJ KANNAN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

For my mother.

ABSTRACT

Sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase (Myoglein; MW 180 kD) is a membrane bound enzyme which requires millimolar concentrations of either Ca^{2+} or Mg^{2+} for maximal hydrolysis of ATP. In order to elucidate the structural and functional properties of the rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase, this protein was purified to homogeneity and utilized for the biophysical, molecular and immunochemical characterization. The biophysical characterization revealed that the isoelectric point (pI) of the cardiac ecto-ATPase was 5.7. The purified Ca^{2+}/Mg^{2+} ecto-ATPase from the rat heart sarcolemmal appeared as a single band with MW ~90 kD in the SDS-PAGE. In order to understand the nature of this enzyme, the 90 kD band in the SDS-PAGE was electroeluted; the analysis of the eluate showed two prominent bands with MW ~90 and 85 kD. The presence of two bands was further confirmed by gradient gel (10-20%) electrophoresis in 0.375 M Tris-HCl buffer, pH 8.8. Analysis of the purified Ca^{2+}/Mg^{2+} ecto-ATPase as well as of the electroeluted protein in a non-equilibrium linear two dimensional electrophoresis (Ampholyte pI 3.0-10.0) also showed two distinct bands. Mass spectroscopic analysis of the enzyme using different matrix combinations revealed the presence of multi-components indicating microheterogeneity in the protein structure. Treatment of the ecto-ATPase with DL-dithiothreitol did not alter the pattern of mass spectroscopic analysis and this indicated that the microheterogeneity may be due to some posttranslational modifications.

For determining the molecular structural properties, the purified Ca^{2+}/Mg^{2+} ecto-ATPase was subjected to tryptic digestion and amino acid sequencing. The amino acid

sequence was utilized to design an oligonucleotide probe. Screening of a rat heart cDNA library produced a partial cDNA clone (pND2.1) that was 100% homologous to human platelet CD36. A high degree of homology (>70%) with other cell adhesion molecules was also noted. The oligomer probe detected a 4.4 kB transcript in heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. An additional 3.1 kB transcript was detectable in heart, lung, liver and skeletal muscle while transcripts of ~2.0 kB and ~1.0 kB were also evident in the heart whereas ND2.1 detected a ~3.1 kB transcript in cardiac tissue. WInterspecies expression analysis (cardiac tissue total RNA blot probed with pND2.1) detected a ~2.0 kB transcript in canine, rabbit and porcine heart whereas transcripts of 4.1 kB, ~3.0 kB, and 2.1 kB were observed in human cardiac tissue. Interspecies (Zoo) Southern blot probed with pND2.1 detected sequences present in human, rat, bovine and yeast. A rat genomic DNA Southern blot, probed with pND2.1, indicated that there was a single copy of the gene in the rat genome. Expression of the pND2.1 cDNA in E. coli produced a protein recognized by anti-human CD36 but not by anti-rat Ca^{2+}/Mg^{2+} ecto-ATPase.

To determine the immunochemical properties of the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase, a polyclonal antiserum was raised against purified Ca^{2+}/Mg^{2+} ecto-ATPase. As assessed by Western blot analysis, the antiserum as well as the purified immunoglobulin were specific for Ca^{2+}/Mg^{2+} ATPase; no crossreaction was observed towards other membrane bound enzymes such as rat sarcoplasmic reticulum Ca^{2+}- pump ATPase or plasma membrane Ca^{2+}-pump ATPase. On the other hand, the cardiac Ca^{2+}/Mg^{2+} ATPase was not recognized by either rat SR Ca^{2+} ATPase or liver ecto-ATPase (Ca^{2+}/Mg^{2+} ATPase) antibodies.

Furthermore, the immune serum was capable of inhibiting the enzymatic activity of the antigen. Indirect immunofluorescence of cardiac tissue sections and neonatal cultured cardiomyocytes with the antibody indicated that the localization of Ca^{2+}/Mg^{2+} ecto-ATPase in association with the plasma membrane of myocytes, in areas of cell-matrix or cell-cell contact. Staining for the ecto-ATPase was not cardiac specific since the antibody detected ATPase in sections from skeletal muscle, brain, liver and kidney.

In this study, we have produced an antiserum which is specific for plasma membrane Ca^{2+}/Mg^{2+} ecto-ATPase in cardiocytes. This antiserum can localize the plasma membrane of cardiomyocytes as well as brain, skeletal muscle, liver and kidney. Western immunoblots revealed that a polyclonal antiserum raised against the purified cardiac Ca^{2+}/Mg^{2+} ecto-ATPase in rabbit recognized the CD36 molecule, whereas a monoclonal antibody directed against human CD36 cross-reacted with cardiac Ca^{2+}/Mg^{2+} ecto-ATPase, in cardiac plasma membrane preparation.

On the basis of these observations, the role of the Ca^{2+}/Mg^{2+} ecto-ATPase in cell adhesion phenomenon was tested by carrying out a cell-cell adhesion bioassay in neonatal cardiomyocyte culture. The purified IgG fraction of the anti-cardiac Ca^{2+}/Mg^{2+} ecto-ATPase serum was found to depress the spreading and attachment of cardiomyocytes to their substratum. These results suggest that the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase is an acidic protein having two subunits. Furthermore, Ca^{2+}/Mg^{2+} ecto-ATPase shows microheterogeneity in its molecular structure. It is concluded that rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase may either contain a fragment with cell adhesion properties or is tightly associated with a protein which is homologous to the adhesion molecule CD36.

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I. REVIEW OF THE LITERATURE

1. Introduction

Adenosine triphosphate (ATP) was discovered in the extracts of the skeletal muscle by Karl Lohmann in Germany and simultaneously by Cyrus Fiske and Yellapragada Subbarow in the United States in 1929 (Lehninger, 1970). When the nutrient molecules such as glucose and fatty acids, are metabolized by oxidation to form the end products CO_2 and H_20, the free energy which becomes available is conserved by coupled synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). The energy dependent activities of the cell are sustained by the energy released from the ATP hydrolysis. In 1941 Fritz Lipmann postulated the unifying concept that "ATP is the primary and universal carrier of chemical energy in cells" (Stryer, 1988). When ATP undergoes hydrolysis with the formation of ADP and Pi the standard free energy change is -7.3 Kcal/mol. The enzyme which catalyses the breakdown of the ATP to form ADP and Pi, has been identified as ATPase in many subcellular organs and plasma membrane. This review summarizes the structural and functional properties of various types of ATPases, such as Ca^{2+}/Mg^{2+} ecto-ATPase, Ca^{2+}-pump ATPase of the plasma membrane, sarcoplasmic reticulum Ca^{2+} ATPase, dynein ATPase, alkaline phosphatase, ATP diphosphohydrolase, Na^+/K^+ ATPase, proton ATPase, actomyosin ATPase, H^+/K^+ ATPase and other ATPases [(1) DnaK/DnaJ/DnaB; (2) P-glycoprotein; (3) Ribosome-associated ATPase; (4) SnF/SwI; (5) RNA-dependent ATPase/helicase; (6) RAD3, protein; (7) ATP-dependent topoisomerase; and (8) ABC transporters/traffic ATPases]. Although, there is a wealth of information

available on the ATPases of different species ranging from prokaryotes to eukaryotes, this review is focused mainly on the eukaryotes with a particular reference to their structural and functional properties.

2. E-type ATPase

A. Ca^{2+}/Mg^{2+} ecto-ATPase

This enzyme has been referred to as Ca^{2+}/Mg^{2+} ATPase or Ca^{2+}-activated ATPase or Mg^{2+}-activated ATPase or plasma membrane E-type ATPase or basal ATPase. Ca^{2+}/Mg^{2+} ecto-ATPase was first identified in 1957 in the liver tissue; however it is now shown to be present on the plasma membrane of different organs and its activation requires millimolar concentrations of either Ca^{2+} or Mg^{2+} for the maximal hydrolysis of its substrate, ATP and several different nucleotide triphosphates (Dhalla and Zhao, 1988; Plesner et al., 1997). Nearly four decades of research on Ca^{2+}/Mg^{2+} ecto-ATPase from different organ systems originating from different species has provided information on its biochemistry and molecular biology without ascribing any definitive physiological functional role to this enzyme (Dhalla and Zhao, 1988; Plesner, 1995; Plesner et al., 1997). The molecular structure of the cell membrane Ca^{2+}/Mg^{2+} ecto-ATPase is not known. The intent of this section is to summarize our present understanding of the structural and functional properties of Ca^{2+}/Mg^{2+} ecto-ATPase in the animal cell membranes.

i. Classification of Ca^{2+}/Mg^{2+} ecto-ATPase

Based on the following criterion, Ca^{2+}/Mg^{2+} ecto-ATPase has been classified as an E-type ATPase [E.C.3.6.1.15] activity (Webb, 1992): i) Ca^{2+}/Mg^{2+} ecto-ATPase has a catalytic

site located on the extracellular surface; ii) Ca^{2+}/Mg^{2+} ecto-ATPase is insensitive to inhibitors of **P-type** ion transporting ATPases [i.e. Na^+/ K^+ ATPase, Ca^{2+}-pump ATPase of the sarcolemma and sarcoplasmic reticulum] such as ouabain (1 mM) and vanadate (50 μM), **F-type** ATPases [i.e. F_1F_0 mitochondrial proton pump] such as NaN_3 (5 mM) and oligomycin (6 μM), and **V-type** ATPases [i.e. vacuolar proton pumps] such as N-ethylmaleimide (NEM, 0.5 mM) and fluoride (10 mM); iii) Ca^{2+}/Mg^{2+} ecto-ATPase is inactivated by detergents; iv) Ca^{2+}/Mg^{2+} ecto-ATPase does not form phosphoprotein intermediates during substrate hydrolysis; v) Ca^{2+}/Mg^{2+} ecto-ATPase is dependant upon millimolar (mM) concentrations of Ca^{2+} or Mg^{2+} as a cofactor; vi) Ca^{2+}/Mg^{2+} ecto-ATPase hydrolyzes nucleoside triphosphates but not nucleoside diphosphates or nucleoside monophosphates; vii) Ca^{2+}/Mg^{2+} ecto-ATPase has an alkaline pH optimum (p7.5 to pH 8.5); viii) Ca^{2+}/Mg^{2+} ecto-ATPase exhibits a high turnover [formation/breakdown] rate with a calculated turnover number of 500,000 min^{-1} (Treuheit et al., 1992); and ix) Ca^{2+}/Mg^{2+} ecto-ATPase is present in low abundance in most tissues (Dhalla and Zhao, 1989; Plesner, 1995; Plesner et al., 1997).

ii. Distinguishing features of Ca^{2+}/Mg^{2+} ecto-ATPase

Ca^{2+}/Mg^{2+} ecto-ATPase has been shown to be distinctly different from other enzymes which are known to hydrolyze ATP (Dhalla et al., 1984; Dhalla and Zhao, 1988).

a. Na^+/K^+ ATPase

Na^+/K^+ ATPase is a marker enzyme for the plasma membrane, and requires Mg^{2+} for its activity. Ouabain, which is a specific inhibitor of Na^+/K^+ ATPase, has no inhibitory effect on Ca^{2+}/Mg^{2+} ecto-ATPase activity of the plasma membranes from different species.

b. Ca^{2+}-pump ATPase

This enzyme is also referred as high affinity Ca^{2+} ATPase or Ca^{2+}-stimulated ATPase and has been shown to be present in the plasma membrane as well as sarcoplasmic reticulum. The Ca^{2+}-stimulated ATPase, which plays a role in efflux of Ca^{2+} requires micromolar concentrations of Ca^{2+} and utilizes MgATP as a substrate.

c. Proton ATPase (F_1F_0-ATPase)

The proton ATPase resides on the inner membrane (submitochondrial particles) of the mitochondria. Mitochondrial ATPase is inhibited by the specific mitochondrial ATPase, inhibitors like oligomycin, DCCD and NaN_3 which have no effect on Ca^{2+}/Mg^{2+} ecto-ATPase.

d. Alkaline phosphatase

Alkaline phosphatase is a non-specific phosphatase which hydrolyzes ATP to ADP and Pi. The Ca^{2+}/Mg^{2+} ecto-ATPase is different from alkaline phosphatase because: i) plasma membrane-bound alkaline phosphatase exhibits optimal activity at pH 10.3 whereas the Ca^{2+}/Mg^{2+} ecto-ATPase is optimally active at pH 7.4-7.5; ii) unlike Ca^{2+}/Mg^{2+} ecto-ATPase, alkaline phosphatase does not require Mg^{2+} or Ca^{2+} as a cofactor for catalytic property. Vanadate, levamisole and cysteine strongly inhibit alkaline phosphatase but have no effect on Ca^{2+}/Mg^{2+} ecto-ATPase activity (Dhalla and Zhao, 1988).

e. ATP diphosphohydrolase

This enzyme catalyzes the hydrolysis of triphosphonucleosides and diphosphonucleosides to yield nucleoside monophosphate and inorganic phosphate

(Knowles et al., 1983). It hydrolyses both nucleoside triphosphate and nucleoside diphosphate at equal rates while Ca^{2+}/Mg^{2+} ecto-ATPase hydrolyzes nucleoside diphosphate at much lower rate in comparison to that of the other nucleoside triphosphate.

iii. Functional properties of Ca^{2+}/Mg^{2+} ecto-ATPase

A variety of functions have been proposed for Ca^{2+}/Mg^{2+} ecto-ATPase (Plesner, 1995; Plesner et al., 1997) including termination of purinergic signalling in smooth muscle cells (Su, 1983), neurotransmission (Burnstock, 1996), non-synaptic information transfer (Trams and Lauter, 1974; Edwards et al., 1992), secretion (Beaudoin et al., 1986; Strobel and Rosenberg, 1992), vesicle trafficking (Kittel and Bacsy, 1994), Ca^{2+}-influx and Mg^{2+}-efflux from cardiac myocytes (Dhalla and Zhao, 1989), regulation of ectokinase substrate concentration (Desouza and Reed, 1991), cell adhesion (Lin and Guidotti, 1989; Cunningham et al., 1993; Cheung et al., 1993; Kannan et al., 1997; Kirley, 1997; Dzhanzhugazyan and Bock, 1993; 1997a, b; Tingstrom et al., 1990; Knowles et al., 1997), bicarbonate transport (Martin and Senior, 1980), tumourigenesis, (Knowles, 1995), mesentric artery functions (Juul et al., 1993), mechanochemical functions involving cell motility and adhesion in HeLa cells and Erlich ascites tumour cells (Epstein and Holt, 1963; Stewart et al., 1969).

iv. Regulation of functional properties of Ca^{2+}/Mg^{2+} ecto-ATPase

Differential regulation of ecto-ATPase activity due to leukocytes were observed during differentiation and maturation (Hehl et al., 1985). Epidermal growth factor-dependent enhancement in ecto-ATPase activity, observed in human hepatoma xenograft requires

hydrocortisone and cholera toxin while the xenograft tissue growth was inhibited (Knowles et al., 1985). Hennighausen and Lange (1985) proposed that the ecto-ATPase activity in rodent thmocytes is related to the maturation of the cells. Based on the inhibitory data obtained from the mammalian brain tissue synaptosomes (rat, mouse, gerbil and human) it was suggested that Mg^{2+}- and Ca^{2+}-dependent ecto-ATPase may represent two different enzymes and they may be regulated independently (Nagy et al., 1986). Still et al. (1986) have shown that the concanavalin (Con A) causes the inactivation of ATPase present in the intact frog skeletal muscles. Mouse macrophage cell line J774 was reported to possess an ATP receptor promoting Ca^{2+}-influx which is limited by the presence of ecto-ATPases for the hydrolysis of ATP (Steinberg and Silverstein, 1987).

It was found that there was no correlation between ecto-ATPase inhibition and contractile force development in guinea pig urinary bladder and vas deferens (Ziganshin et al., 1995). In human brain, the posterior part of epileptic hippocampus has been shown to exhibit a marked increase in the ecto-ATPase activity, which has been suggested to be involved in the epileptic seizure development and sustenance of epilepsy in human brain (Nagy et al., 1990). It has been shown that after the stretch injury in the nerve fibers, the ecto-ATPase was lost from the external side of the myelin sheath where the focal separation of myelin lamellae occurs; this event occurs 1 and 4 hr after the injury and has been characterized as a loss of regular axonal structure (Maxwell et al., 1995). It has been reported that ecto-ATPase inactivation occurs after the ATP hydrolysis in brain synaptosomes. However ATP hydrolytic products, ADP, AMP, adenosine and inorganic

phosphates did not affect the ecto-ATPase inactivation. Further the concanavalin A confers partial protection to ATP hydrolysis-induced inactivation of ecto-ATPase. It was suggested that the ecto-ATPase inactivation is partly due to the phosphorylation of membrane-bound proteins (Romero et al., 1996).

The adenosine nucleosides (ATP, ADP, AMP and adenosine) have been shown to stimulate the proliferation of the endothelial LLC-MK2 cells. Whereas the inhibition of the ecto-ATPase, 5'-nucleotidase or alkaline phosphatase reduced the proliferation of the LLC-MK2 cells. Thus, it was suggested that the different purines and pyrimidines may contribute to the proliferation of the LLC-MK2 cells (Lemmens et al., 1996). A novel purinoceptor (P_1), where both adenosine and ATP act as agonists, was found to be present on the surface of follicular oocytes of Xenopus laevis. Since the ecto-ATPase confers low rate of ATP hydrolysis, the ecto-ATPase does not seem to generate sufficient ligand (Ado/AMP) to function as a P_1 purinoceptor (King et al., 1996).

v. Structural properties of the Ca^{2+}/Mg^{2+} ecto-ATPase

The Western (immuno)blots with polyclonal antibodies for Ca^{2+}/Mg^{2+} ecto-ATPase indicated that the liver Ca^{2+}/Mg^{2+} ecto-ATPase was more enriched in the canalicular domain (Lin, 1989). Three different anti-Ca^{2+}/Mg^{2+} ecto-ATPase antibodies have been reported. The antibody #669 raised against SDS-PAGE purified Ca^{2+}/Mg^{2+} ecto-ATPase, only recognized the denatured Ca^{2+}/Mg^{2+} ecto-ATPase. Antibody #708 was generated for chromatographically purified enzyme (i.e. non-denatured protein), and recognized both the native and denatured Ca^{2+}/Mg^{2+} ecto-ATPase. This antibody has been extensively employed

in immunoprecipitation analysis (Lin, 1989). Antipeptide #36 was generated against a sequence obtained from tryptic digestion of purified liver Ca^{2+}/Mg^{2+} ecto-ATPase. It recognized the reduced form of Ca^{2+}/Mg^{2+} ecto-ATPase but not the non-reduced form (Buxton et al., 1986; Lin, 1990). A rat liver hepatocyte protein with Mr 100,000 has been characterized as Ca^{2+}/Mg^{2+} ecto-ATPase. It is present in two isoforms with different C terminals viz 1O AA and 7O AA, while the ATP binding domain localized on the extracellular domain. Furthermore, sequence homology analysis of the liver ecto-ATPase showed that it has three immunoglobulin-like domains that are homologous to those that are present in the biliary glycoprotein-1 (BGP1). There are 16 potential asparagine-linked N-glycosylation sites in this protein (Lin et al., 1991).

The primary structure of the rat liver plasma membrane ecto-ATPase has the nucleotide and amino acid sequence homology with human biliary glycoprotein I. However it should be noted that this cDNA sequence has no Walker motives (I $GX_1X_2X_3X_4$-GK or II R/K-$X_1X_2X_3$-L; X = hydrophobic residues) which represents the ATP binding domain in the ion motive ATPases. The lack of Walker ATP binding domain sequence was attributed to the insensitivity of ecto-ATPase's to vanadate, which has been identified as a characteristic feature of the ion motive ATPases to form aspartyl phosphoprotein intermediates (Lin and Guidotti, 1989). However, the rat liver Ca^{2+}/Mg^{2+} ecto-ATPase cDNA has been shown to be detecting the transcripts of a biliary glycoprotein and carcinoembryonic antigen, but not the mercurial insensitive ecto-ATPase in small cell lung cancer cell lines (Knowles, 1995). Further, the expression product of the liver ecto-ATPase cDNA has been shown to be

functioning as a cell adhesion molecule without E-type ATPase (ecto-ATPase) activity (Cheung et al., 1993).

Hydropathy plot analysis of rat liver ecto-ATPase cDNA sequence showed that it has two hydrophobic stretches. One is located at the NH_2-terminal and forms part of a membrane signal sequence and the other is located near the COOH-terminal end. The overall structural arrangement (amino acid) predicts that most of the liver ecto-ATPase protein mass is in the extracellular space and that the COOH terminal of the protein is intracellular. This structural information is consistent with the fact that the liver Ca^{2+}/Mg^{2+} ATPase protein is an ecto-ATPase (Lin, 1990). The two stretches of the amino acid sequence (consisting of amino acids 92-100 and 335-348) are similar to the consensus sequences for nucleotide-binding domains of other ATP-binding proteins. The expression construct containing the coding region of the rat liver Ca^{2+}/Mg^{2+} ecto-ATPase, cloned in front of the cytomegalovirus promotor and SV 40 origin of replication (pEXP), was expressed in the mouse L cell line and the HeLa cell line. The lysate obtained from the cells transfected with the plasmid pEXP had a higher Ca^{2+}-activated ATPase activity compared to that of the control (Lin, 1990). There are two rat liver cell-CAM 105 isoforms (long and short) which have been cloned and characterized. The short isoform is predominant on the external surface of the rat liver plasma membrane. The longer isoform has more potential phosphorylation sites than the shorter isoform. Differential phosphorylation could be one of the mechanism for differential isoform function. Both long and short isoforms are localized in the canalicular domain of hepatocytes. The difference in the sequence of the two

isoform suggests that these are probably derived from different genes rather than being formed by alternative splicing (Culic et al., 1992). Mg^{2+} ATPase (Mr 85 kD; glycoprotein), purified from the traverse tubules of chicken skeletal muscle, has been shown to have the amino acid sequence homologous to T cadherin, which shares many biochemical properties of traverse tubule Mg^{2+} ATPase of chicken skeletal muscle (Cunninhgam et al., 1993).

The ectonucleotidase activity profile (ecto-ATPase; ecto-ADPase) of human umbilical vein endothelial cells was similar to that of the enzyme from leukocytes (Coade and Pearson, 1989). Fillipini et al. (1990) postulated that the presence of ecto-ATPase on the cytolytic thymus derived lymphocytes confers protection from the deleterious effect mediated by extracellular ATP. An antiserum directed against bovine aortic endothelial smooth muscle cell ATP diphosphohydrolase showed marked inhibition on both ecto-ATPase and ecto-ADPase of bovine aortic endothelial smooth muscle cells. It was suggested that both enzyme activities are probably conferred by one enzyme referred to as ATP diphosphohydrolase (Yagi et al., 1991). Sequential hydrolysis of ATP and ADP in the adult rat cerebral cortex synaptosomes was attributed to the presence of an ATP diphosphohydrolase (Battastini et al., 1991). An ecto-ATPase requiring mM concentration of Ca^{2+} or Mg^{2+} has been localized on the taste bud of the cells and it is suggested to be involved either in neurotransmission or in the energy supply mechanism in the taste bud function (Barry, 1992).

Kurihara et al. (1992) demonstrated that the ecto-ATPases hydrolyzing nucleoside triphosphates in A-431(epidermal carcinoma) cells, were different from P_2 purinergic

receptors. Based on in vitro studies on ecto-ATPase (Mg^{2+} or Ca^{2+}-dependent ecto-ATPase) in Langerhans cells, it was suggested that this mATPase confers protection against membrane lytic effect mediated by extracellular ATP $[ATP]_o$ (Giolomoni et al., 1992). Beukers et al. (1993) demonstrated that human platelets possess ecto-ATPase with high affinity for ATP, as well as for ADP which causes the platelet aggregation. It was thus suggested that the role of ecto-ATPase activity in platelet aggregation may be minimal because breakdown of ATP to ADP occurs slowly. 8-Azido-ATP (8-N3-ATP) has been shown to be a substrate for ecto-ATPase in cultures of chromaffin cells with a Km of ~256.30 mM and Vmax of 14.33, (Rodriguez-Pascual et al., 1993). An ATPase isolated from bovine synaptosome membranes with Mr 50 kD was considered as an ecto-ATPase due to the fact that the active site was exposed to external environment (Homan et al., 1993). It has been demonstrated that Ca^{2+}/Mg^{2+} ecto-ATPase activity is associated with neural cell adhesion molecule purified from rat brain microsomes (Dahandzhugazyan and Bock, 1993). However, it has been shown that the Mg^{2+} ATPase activity and NCAM could be separated in the partially purified rat and chicken brain microsomes (Stout et al., 1994).

Ca^{2+} or Mg^{2+}-activated ATPase has been isolated from the bovine brain synaptosomal plasma membranes. It has a Mr 5O kD and pI of 5.3 to 5.9; it requires millimolar concentration of Mg^{2+} or Ca^{2+} for maximal hydrolysis with an optimum pH between 7.5 and 8.5. As the active site of the membrane-bound enzyme faced the extracellular medium, it was considered to be an ecto-ATPase (Hohman et al., 1993). Sippel et al. (1993) showed that bile acid efflux and Ca^{2+}/Mg^{2+} ecto-ATPase activities were two distinct properties of a

single rat liver hepatocyte canalicular membrane protein. However, introduction of mutations in the consensus sequence at amino acids Gly 97 and Arg 98 in the Ca^{2+}/Mg^{2+} ecto-ATPase abrogated ATPase activity but did not affect its bile acid transport activity (Sippel et al., 1994). Kast et al. (1994) reported that electrogenic taurocholate transport resides entirely in the endoplasmic reticulum, whereas ATP-dependent bile acid transport is an intrinsic function of the canalicular membrane as well as a yet unidentified intracellular membrane-bound compartment. Therefore the two transport activities were most probably mediated by two different bile acid transporting polypeptides.

The Ca^{2+}/Mg^{2+} ecto-ATPase has been suggested to be a receptor for ATP and ADP. The activation of Ca^{2+}/Mg^{2+} ecto-ATPase stimulated nitric oxide secretion in cultured bovine endothelial cells. Whereas inhibitors of Ca^{2+}/Mg^{2+} ecto-ATPase, such as sodium azide and Ap5A [Adenylate Kinase inhibitor,→ P^1, P^5-Di(Adenosine-5') pentaphosphate], significantly inhibited the nitric oxide secretion. Also, RB2 [Reactive Blue-2], an inhibitor of P_2Y purinoceptor and Ca^{2+}/Mg^{2+} ecto-ATPase, completely suppressed the nitric oxide secretion (Yagi et al., 1994). An antipeptide antibody [N-terminal amino acid sequence of the chicken gizzard Ca^{2+}/Mg^{2+} ecto-ATPase = KILSGEEEGVFG] identified that chicken gizzard Ca^{2+}/Mg^{2+} ecto-ATPase and T-Cadherin are different (Stout and Kirley, 1994). It should be mentioned that Ca^{2+}/Mg^{2+} ecto-ATPase was localized on the luminal surfaces and intercellular canaliculi of the acinar cells of both in rat parotid and submandibular glands. Immunogold labelling was also observed on the luminal borders of the intercalated and striated ducts. It was suggested the C-CAM may play a major role in ATP-breakdown,

stabilization of microvillar membranes, cell adhesion and involvement in secretory mechanisms in the rat parotid and submandibular glands (Murphy et al., 1994).

Rat hepatocytes were shown to possess both mercurial sensitive (p-chloro mercuri phenyl sulfonate; [pCMPS]) and mercurial insensitive ecto-ATPase. During the hepatoma (hepatoma tumour) formation, the mercurial sensitive ecto-ATPase is increased whereas the latter is decreased. It was also found that the mercurial-sensitive ecto-ATPase was also expressed at high level in three lines of human small cell lung carcinoma (SCLC) cells compared to the normal cells (Shi and Knowles, 1994).

When the rat glomerular mesangial cells were cultured without serum, a reduction in the ecto-ATPase activity was evident (Stefanovic et al., 1995). A unique, ecto-ATPase partly sensitive to DCCD (dicyclohexylcarbodiimide) was demonstrated to be present on the surface of the human placental brush border membranes (Brunette et al., 1995). Suramin, Ni^{2+} and ATPγ(S), showed inhibitory effect on the human blood cells ecto-ATPase activity (Beaukers et al., 1995). By utilizing specific monoclonal antibodies, an ecto-ATPase protein was immunoprecipitated. This protein was shown to have amino acid sequence homology with integrins a group of proteins involved in the contact between the cell adhesion molecules (Stout et al., 1995).

An ecto-ATPase localized on the rat small intestinal brush border membrane was proposed to play a major role in the nutrient break down (Schweickhardt et al., 1995). It has been shown that the purinergic receptor antagonists (PPADS, suramin, and reactive blue) inhibited the ecto-ATPase activity in the bovine pulmonary artery endothelium, glial cella

and macrophages (Chen et al., 1996). A human plasma factor 100 KF, with reduced expression of ecto-ATPase, has been shown to increase the permeability of human endothelium for macromolecules in a dose-dependent fashion (Cheung et al., 1996). Alternate perfusion of the rat kidney (ex vivo) with plasma protein factor 1100 KF resulted in a significant loss of glomerular polyanion (GPA), as well as glomerular ecto-ATPase. The ability of 100KF to induce minimal change like glomerular lesions, in association with selectively increased permeability for plasma proteins, suggests that this human plasma constituent may be important in the pathogenesis of this disease (Cheung et al., 1996).

The partially purified rat liver plasma membrane and lysosomal Ca^{2+}/Mg^{2+} ATPases have been shown to have similarity to that of an ecto-ATPase in the following characteristics: broad pH-activity profiles, K_m values for ATP being 21-27 μM (at pH 4.5) and 18-14 μM (at pH 7.0) (in the presence of Ca^{2+}), hydrolysis of both ATP and ADP, inhibition only by vanadate and 4,4'-diisothiocyanatostilbene 2,2'-disulfonic acid (DIDS), acidic pI values, and crossreactivity against an antibody to the N-terminal peptide of ecto-ATPase (Adachi et al., 1996). By inhibiting the ecto-ATPase activity by an ecto-ATPase inhibitor, ARL67156 [6-N,N-diethyl-D-β,γ,-dibromo methylene ATP], it has been shown that there was an increase in nerve stimulation and neurogenic contractions in the isolated guinea pig vas deferens (Westfall et al.,1996). Based on the observations such as differences in the pH, divalent cation requirement, and the absence of inhibitory effect of ADP on degradation of ATP during ATP hydrolysis on the surface of the oocytes of Xenopus laevis, it was suggested that the degradation of ATP and ADP are mediated by two separate Ca^{2+}-

or Mg^{2+}-dependent ecto-ATPase and Ca^{2+}/Mg^{2+}-dependent ecto-ADPase (Ziganshin et al.,1996).

Following demyelination there was a continuous expression of the ecto-ATPase activity in the internodal axolemma of exposed axons, and specifically the ecto-ATPase activity was prominent at the sites of axonal contacts and glial-cell processes. However, when remyelination takes place, the area of the axonal surface exhibiting the ecto-ATPase activity decreased in direct proportion to the thickening of the new myelin sheath. Thus the differential expression of the ecto-ATPase activity significantly plays a role in the axo-glial recognition and founding of axo-glial contacts (Felts and Smith, 1996). The crosslinking studies performed with the agents 3,3'-dithiobis (sulfosuccin imidylpropionate) (DTSSP) and dithiobis (succinimidylpropionate) (DSP) on chicken gizzard smooth muscle ecto-ATPase resulted an oligomeric state (130 kD immunoreactive band) causing an increase in the ecto-ATPase activity. Whereas the destabilizing agents proposed to inhibit the ecto-ATPase activity showed reduced ecto-ATPase activity (Stout and Kirley,1996).

A novel membrane associated ecto-ATPase was identified in rat Sertoli cells. This enzyme was considered to be an cell ecto-ATPase due to the fact that it was activated by either Mg^{2+}or Ca^{2+} , and it hydrolyzed other nucleoside triphosphates, but not ADP (Barbacci et al.,1996).A soluble E-type ecto-ATPase was also purified from the single-celled eukaryote, Tetrahymena (Smith et al.,1997). This enzyme shared immunocrossreactivity with the membrane-bound chicken gizzard smooth muscle ecto-ATPase (66 kDa) and a 66-kDa protein in Tetrahymena plasma membranes. This ecto-ATPase enzyme was proposed to act

as an inactivator of the purinergic signals (chemorepulsion responses to extracellular ATP and GTP) and also in the clearance of the extracellular nucleotides. In addition to the previous findings, it has been conclusively demonstrated that besides the ecto-ATPase there are other proteins, which are involved in the ATP- dependent bile acid transport into the bile in rat hepatocytes, rat hepatoma HTC cells, and HTC (HTC-R) cell lines (Luther et al., 1997). Apyrase, an enzyme with ecto-ATPase activity, abolished the ATP-mediated cytotoxicity in the human tumor cell (LoVoDx cells) (Correale et al., 1997).

Chicken gizzard smooth muscle ecto-ATPase cDNA sequence has considerable sequence homology with mouse and human CD39, excluding the membrane spanning region, and also it was suggested to be involved in the homotypic cell adhesion process (Kirley, 1997). CD39 is a lymphoid cell (B cell) differentiation marker present in the Epstein Barr virus transformed immunocompetent cells and this molecule has been shown to have apyrase activity. The CD39 molecule was demonstrated to be an ecto-Ca^{2+}- or -Mg^{2+}-dependent apyrase (Wang and Guidotti, 1996).

It has been suggested that E-type ATPase activity may be tightly bound to proteins having adhesion characteristics. This conclusion was based on studies conducted on both ecto-ATPases and cell adhesion molecules. It was observed that the sequence for the ecto-ATPase purified from rat liver is homologous to the human biliary glycoprotein I (Lin and Guidotti, 1989). Furthermore, it was noted that the liver ecto-ATPase cDNA sequence contains neither of the Walker motives (I: $GX_1X_2X_3X_4$-GK or II: R/K-$X_1X_2X_3$-L-, where X = hydrophobic residues) representative of the ATP binding region of ion transporting

ATPases (Lin and Guidotti, 1989). While the absence of a Walker motif may indicate this protein is not capable of ATP hydrolysis, this feature is characteristic for ion motive ATPases forming phosphoprotein intermediates (Shull and Greeb, 1988) and the rat liver plasma membrane high affinity Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase is insensitive to vanadate. It is implied that aspartyl phosphate intermediate formation is not required for ATP hydrolysis by rat liver ecto-ATPase (Lin, 1985).

Expression of the rat liver ecto-ATPase cDNA, however, demonstrated that this clone coded for a protein in *E. coli* which had the properties of a cell adhesion protein but it lacked the ATPase activity (Cheung et al., 1993). It may nevertheless be possible that this particular isoform of liver ecto-ATPase has an activity too low for the detection due to inefficient folding in the bacterial cell. Similar to the rat liver enzyme, cloning of the chicken gizzard smooth muscle ecto-ATPase produced a cDNA with a sequence that has considerable sequence homology with mouse and human CD39 (Kirley, 1997). Interestingly, CD39 is a cell adhesion protein that has been found to have an ecto-Ca^{2+}/Mg^{2+} apyrase activity capable of hydrolyzing both ATP and ADP (Wang and Guidotti, 1996; Kirley, 1997; Beaudoin et al., 1997). Expression of the chicken gizzard smooth muscle ecto-ATPase cDNA has yet to be reported and until the expression product has been functionally characterized, the identification of the cDNA for ecto-ATPase remains impossible.

While there is no direct or indirect evidence to prove or disprove that ecto-ATPase/E-type ATPase activity is associated with adhesion molecules, it is intriguing to note that β_2 subunit of the Na^+/K^+ ATPase possesses homology with cell adhesion

molecule of glia (Gloor et al., 1990). Similarly, ecto 5' nucleotidase (hydrolyzes AMP to adenosine) [E.C.3.1.3.5] has also been demonstrated to harbor and/or function as an adhesion molecule, CD73 (Airas and Jalkanen, 1996). Thus, there is considerable evidence to indicate E-type ATPases are directly or indirectly associated with the cell adhesion proteins. As was noted for CD39, the converse relationship is also possible. Proteins involved in adhesion (eg. NCAM, C-CAM, CD36, CD39) may possess an inherent extracellular ATP binding domain capable of hydrolyzing this nucleoside triphosphate. Adhesion molecules exhibiting this property are insensitive to inhibitors of P-type, V-type and F-type ATPases (Plesner et al., 1997). While evidence for the close relationship between E-type ATPases and cell adhesion molecules was documented by several research groups (Lin and Guidotti, 1989; Cunningham et al., 1993; Cheung et al., 1993; Dzhandhugazyan and Bock, 1993; 1997a,b; Knowles, 1995; Kirley, 1997; Kannan et al.,1997), an accurate description of how these distinct activities are coupled has yet to be attained. Resolution of this issue remains controversial as demonstrated by the divergent conclusions reached with respect to NCAM: Stout et al. (1994) demonstrated NCAM can be physically separated from an ecto-Mg^{2+} ATPase activity by anion exchange chromatography, while Dzhanzhygazyan and Bock (1997b) showed that NCAM can be affinity labelled with an ATP analogue.

The purinergic receptor antagonists ATPγS, α,β-methylene ATP (α,β, MeATP) and AMP-PNP have also been shown to exert an inhibitory effect on the ecto-ATPase in the bovine pulmonary endothelial cells, with IC 50 values of 5.2, 4.5 and 4.0; this inhibitory effect was attributed to their potent purinergic antagonists activity (Chen et al., 1997). In

summary, even though several attempts were made to obtain and characterize the pure mammalian Ca^{2+}/Mg^{2+} ecto-ATPase, a careful analysis of the available literature indicates that its precise molecular structure remains unknown.

3. F-type ATPase

A. Mitochondrial F_1F_0 ATPase

This enzyme has also been referred to as H^+ ATPase (F_1F_0 ATPase). Although there is a wealth of the information available on the structure and functional role of this enzyme, some structural properties with reference to alterations in the function of ATP synthase are presented in this section.

i. Structural properties of F_1F_0 ATPase

The F_1F_0 ATPase or ATP synthase is made up of F_1 and F_0 subunits. The F_1 is a catalytic subunit mediating the synthesis of ATP is made up of 5 polypeptide with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ with Mr 378 kD. The F_0 subunit is hydrophobic in nature, functioning as proton channel of this enzyme and is made up of six polypeptide chains, each with Mr 8 kD forming the pore for the transport of protons. An oligomycin-sensitive component is present in the stalk region between F_1 and F_0 ATPase. This component inhibits ATP synthesis by interrupting the proton consumption (Stryer, 1988). Nile blue A (2 μM), pyronin Y (8 μM), acridine orange (25 μM) and coriphosphine (75 μM) stimulated the F_1 ATPase activity (Mai and Allison, 1983). Subunit 9 of mitochondrial F_1F_0 ATPase is a proteolipid and it is essential for proton translocation and it codes for 76 amino acids (Ooi et al., 1988). Substitution of amino acid at unc 513-c subunit of F_1F_0 ATPase by threonine

results in normal assembly but partial functional role in oxidative phosphorylation. Treatment of the F_1F_0 ATPase with DCHC (dicyclohexyl carbodiimide) results in the loss of ATPase activity (Fimmel et al., 1985).

Mutation in B subunit of F_1F_0 ATPase, Gly9→Asp and the complementary suppressor mutant pro240→leu results in a complex F_1F_0 which lacked binding of F_1 to F_0; however, the F_0 composition was normal (Vik and Simoni, 1987). It has been observed that the mitochondrial F_1 component with ATPase activity was inactivated by the presence of Mg^{2+} in the assay medium. The underlying mechanism described was that α and γ components were disassociated in the F_1 subunit. Further reconstitution of F_1 and F_0 in to an enzyme complex leads to the formation of active enzyme complex protected against divalent cation inactivation (Pederson et al., 1987). Structure-function relationship of *E. coli* F_1F_0 ATPase complex was determined by limited tryptic digestion. Trypsin digestion of F_1F_0 complex resulted in 6-8 fold increase in ATPase activity depending on the enzyme preparation. α,β subunit remained in the complex along with γ subunit with high ATPase activity (Gavilanes et al., 1988). Substitution of amino acids in α Glu196 by Asp or Gln or His or Asn or Lys or Ala or Ser or Pro abolishes the ATP driven proton translocation and proton permeability by F_0 subunit. α 190 substitution by Glu or Arg impairs the F_1F_0 ATPase assembly and eliminates proton translocation by ATP (Vik et al., 1988). Substitutions of amino acids in *E. coli* F_1 ATPase in β S339Y and β S339F, abrogated the ATPase activity as well as F_1F_0 ATPase assembly. This observation is consistent with the X ray crystallographic data where F_1 catalytic domain is unoccupied and appears as a cleft; β S339

is present in the cleft (Abrams et al., 1994; Schmidt and Senior, 1995).

F_1F_0-antibody complex formation yielded information on the orientation of F_1F_0 during F_1F_0-mediated ATP hydrolysis. From this study it was concluded that F_1 does not rotate around F_0 during ATP hydrolysis (Gautheron and Godinot, 1988). Substitution of F_1F_0 subunit C amino acid residue 25 alanine to tyrosine resulted in a low level of ATPase activity and total loss of proton transport (Fimmel and Fordham, 1989). The bovine mitochondrial F_1 ATPase activity was inhibited by the cationic and amphiphilic peptides such as bee venom peptide, meltitin (a synthetic peptide) and yeast cytochrome oxidase subunit IV. Amphiphilic peptides showed non competitive inhibition whereas rhodamine 6G; vosaniline, malachite green, coriphosphine, acridine orange and syn C exhibited mixed inhibition (Roise et al., 1988). $\alpha 3,\beta 3$ complex of F_1 ATPase has been shown to be the domain responsible for catalytic property of mitochondrial F_1 ATPase enzyme (Miwa and Yoshida, 1989). Amino acid substitutions in the second, third and fourth transmembrane domains of subunit F_1 (Asp119 → His; Ser → Phe or Gly → Arg) resulted in reduced ATPase activity leading to the reduced H^+ translocation but no impact on the F_0 subunit assembly (Paule and Fillingame, 1989). Substitution of Tyr344→Ala 344 of beta subunit of yeast mitochondrial F_1 ATPase resulted inactive form in vivo (Mueller, 1990). ATP hydrolysis by F_1 subunit in solution occurs when it is attached to the inner mitochondrial membrane only. This hydrolysis event is promoted by ATP binding to two other active sites of F_1F_0 complex (Wu et al., 1990). For the optimal H^+ (proton) transport by F_1F_0 ATP synthase, the Asp 24 residue at position 61 has been shown to be protonated in subunit C (Zhang and Fillingame,

1994).

ii. Functional properties of F_1F_0 ATPase

Amino acid substitutions at Gly29→Val in C subunit resulted low levels of ATPase activity and also low permeability in proton transport (Fimmel and Norris, 1989). Substitution of amino acid at Asp124 → asparagine and Arg140 → glutamine in α subunit of F_1 ATPase resulted in an altered in the subunit structure and the loss of ATP synthase function. The underlying mechanism was hypothesized to be that Asp 124 forms a salt bridge with Arg140 which plays a role in a subunit structure (Howitt et al., 1990). Interaction of proton (H^+) transport machinery in F_0 and the catalytic site in F_1 component of the F_1F_0 ATPase are prerequisite for energy coupling (Senior, 1990). Nuclear genes ATP_{11} and ATP_{12} of pet mutant [nuclear respiratory deficient mutants of *Saccharomyces cerevisiae*] have been shown to play a crucial role in the assembly of F_1 part of F_1F_0 ATPase (Ackerman and Tzaagoloff, 1990). Fluorometal combination (fluoride-aluminium or berellium)-ADP inhibits the beef cardiac mitochondrial F_1F_0 ATPase. ADP-fluorometal complex binds to F_1 subunit in a partially irreversible manner (Issartel et al., 1991).

Amino acid residues 137-335 of β subunit in F_1 ATPase was suggested to be the catalytic site of the enzyme (Senior, 1992). The ϵ subunit of F_1F_0 ATPase of *E. coli* has been crystalized to 2.9 A (Codd et al., 1992). Further the conserved acidic residues (Asp61) in C terminal of subunit C is a prime requirement for F_0 to bind to F_1 subunit (Deckers-Hebestreti and Altendorf,1992). An inhibitor protein for ATP synthase/ATP complex has been cloned and sequenced. It has 25 amino acid in the N-terminal sequence and is required

for mitochondrial targetting. It has nucleotide sequence homology with bovine cardiac mitochondrial F_1F_0 ATPase (~70%); and yeast inhibitor protein (~40%) (Lebowitz and Pederson, 1993). The residues ser 174 and Ala 295 → Pro reversions is crucial for F_1F_0 ATPase mediated catalysis (Miki et al., 1994). Structural integrity of hydrophobic domain of ATP synthase (subunit b) is an important factor for the optimal functioning of the proton transport and ATP synthesis in yeast mitochondria (Razaka et al., 1994).

It has been shown that the alanine substitution in the 10 and 93 residue of *E. coli*, F_1F_0 ATP synthase ϵ subunit, showed that binding of epsilon to other subunit is inhibited. Substitution of Glu 59 → Glu 70 is required for the release of epsilon mediated inhibitions (Xiang and Vik, 1995). Atp11p protein is a principle requirement for F_1 assembly in *S. cerevisiae* mitochondrial ATP SYNTHASE. The sequence between Phe120 → Asn174 was identified as an active domain. Residues near (Glu40 → Ser 109) and Arg 183 → Asn318 of the active domain was identified as prime requirement for the F_1F_0 ATPase structural stability in the mitochondrial milieu (Wang and Ackerman, 1996). It was suggested that unfolding of natural inhibitor protein is a prime requisite for optimal inhibitor function on F_1 component of mitochondrial ATP synthase. Also, pH reduction from pH 8.0 → pH 6.0 caused increase in ATPase inhibition by its inhibitors (Lebowitz and Pederson, 1996). F_1F_0 ATPase isolated from *Enterococcus hirae* (ATCC 9796) functions as a regulator of the cytoplasmic pH but does not synthesize ATP. One transcript codes for the subunits a, c, b, δ, γ, β, ϵ. The deduced amino acid indicates that *E. hirae* H^+ ATPase is a typical F_1F_0 type ATPase. However, its gene structure is identical to that of other bacterial F_1F_0

ATPase (Shibata et al., 1992).

A mutation in the wild type transmembrane-cysteine replaced by tyrosine (Y) or phenylalanine (F) increased the inhibition constant (Ki) of ouabain greater than 1000 fold compared with its wild type. This indicates that the transmembrane segment participates in the structure and function of the ouabain binding site (Canessa et al., 1994). Oligomycin sensitivity conferral protein, coupling factor 6 (F_6) and subunits b,d of bovine mitochondria have been over expressed in *E. coli* and allowed to reconstitute in the presence of F_1 ATPase. Binding of various subunits to F_1 ATPase increases the ATP hydrolytic activity and diminishes its activation by exposure to cold, demonstrating intersubunit interaction in the stalk region of the F_1F_0 ATPase complex (Collinson et al., 1994). A yeast ATP_3 coding for γ subunit has been cloned. It has a dual function involving catalysis of hydrolysis and synthesis as well as in the assembly/stability of F_1 ATPase (Paul et al., 1994).

4. P-type ATPase

A. Ca^{2+}-pump ATPase

This enzyme is also referred to as high affinity Ca^{2+} ATPase or Ca^{2+}-pump ATPase. The Ca^{2+}-stimulated ATPase is present on the plasma membranes of all eukaryotic cells. Ca^{2+}-pump ATPase, has been shown to transport Ca^{2+} out of the erythrocytes (Schatzmann, 1966). This pump has also been shown to be present in the plasma membranes of heart and nerve cells, where an active Na^+/Ca^{2+} exchanger is also present to extrude Ca^{2+} (Baker et al., 1967; Reuter and Seitz, 1968).

i. Structural properties of plasma membrane Ca^{2+}-pump ATPase

The general mechanism of the action of Ca^{2+}-pump follows the pattern of all other P-type ion pumps. ATP phosphorylates an aspartic acid residue in a Ca^{2+}-dependent manner. The phosphorylated protein has a molecular mass of about 140 kD and has high affinity for Ca^{2+}. A stoichiometry approaching two has also been measured (Sarkadi et al., 1977). The step in the reaction cycle during which the translocation of Ca^{2+} across the membrane occurs is still unknown (Carafoli, 1991). The plasma membrane Ca^{2+}-pump from the erythrocyte membrane has been purified and reconstituted into liposomes where it was shown to transport Ca^{2+} with stoichiometry to the ATP approaching one (Niggli et al., 1979). The complementary DNA coding for the calcium pump has been cloned from rat brain (Shull and Greeb, 1988) and from human teratoma (Verma et al., 1988). There are two isoforms of the pump having 1176 and 1198 amino acids. The human cDNA library has produced one isoform containing 1220 amino acids, which is over 99% identical to the rat isoform in the first 1117 residues but differs substantially in the c-terminal domain. The first domain connecting the transmembrane helices two and three contains helical and antiparallel β sheets domains. The unique sequence responsive to the acidic phospholipids found in the plasma membrane Ca^{2+}-pump, is present in the N-terminal region of the pump (Zvitch et al., 1990).

The second regulatory domain protrudes from transmembrane helix ten with about 160 residues and contains the calmodulin-binding regions (residues 1086 to 1115) which phosphorylates a ser 1178 residue, which is located about 50 residues away from the C-terminus of the pump. The major unit protruding into the intracellular space about 430

residues connects transmembrane helices four and five and is predominantly arranged in alpha-helices and parallel beta sheets. It contains the aspartyl-phosphate residue (Asp 475) in the human teratoma isoform and a domain called the hinge which is generally conserved in all P-type ion pumps and is postulated to bring asp 475 and lys 609 close together during the catalytic cycle (McLennan et al., 1985). The calmodulin binding domain flanked by two acidic stretches would be good candidate to bind Ca^{2+} (Verma et al., 1988). A monoclonal antibody directed against human red blood cell Ca^{2+} pump ATPase localized one of its epitope of Ca^{2+} pump ATPase in osteoblast-like cells. Paraffin-embedded osteoblast-like cell sections demonstrated anti-Ca^{2+}-pump ATPase staining in plasma membrane. In immunoblots it was identified that a 40 kD plasma membrane-bound Ca^{2+}-pump ATPase which is similar to the plasma membrane Ca^{2+}-pump ATPase. These observations raise the possibility that Ca^{2+}-pump ATPase may play a role in the osteoblast intracellular calcium homeostasis (Borke et al., 1988).

Recently a second isoform has been found to be expressed in human erythrocytes in addition to the first isoform found in human teratoma cells (Strehler et al., 1990). It contains 1205 amino acids, shows about 75% identity with the first human isoform, and appears to be the major erythrocyte isoform. It lacks the sequence that is phosphorylated by the cAMP-dependent protein kinase. The sequence of yet another isoform has been deduced from complementary cDNA in rat brain (Shull and Greeb, 1989). The alternative splicing of human plasma membrane Ca^{2+}-pump (PMCA) genes reveals a differential expression of PMCA. The structures of the involved exons appear to be similar in both human teratoma

cells and rat brain with one exception being the PMCA2 gene in rats (Adamo and Penniston, 1992). The situation is more complex at site C. Three different isoforms have been found for PMCA2 in rats (Keeton et al., 1993). The pattern for the PMCA1 splicing at site C has already been described (Strehler et al., 1989). Four transcripts can be generated at site C. Three of them use internal acceptor-donor sites of the same exon. Only two alternatively spliced transcripts have been detected for PMCA4 (Brandt et al., 1992a). The results for PMCA3 were more complex: that is two exons with internal acceptor-donor sites are involved in the generation of six possible alternatively spliced mRNAs (Keeton et al., 1993). Only four of these alternatively spliced mRNAs however have been detected in humans. Site A occurs immediately to the N-terminal of the putative phospholipid binding domain (Zvaritch et al., 1990).

Site C is located at the end of the calmodulin-binding region and has been shown to influence the binding of calmodulin (Kessler et al., 1992). The expression of the calcium pump regions encompassing the calmodulin-binding domain and the regions derived from alternative splicing at site C (Kessler et al., 1992). Results on the bacterially expressed C-terminus of the isoform 1b have shown that its ability to interact with calmodulin is not influenced by pH. By contrast, the corresponding regions of isoforms 1a/1d/1c, whose spliced-in sequences contain histidines, exhibit pH-dependent calmodulin-binding (Kessler et al., 1992). Since these isoforms have been detected in muscle, the pH changes during heavy muscular work could in principle influence the activity of the pump through modified calmodulin binding. The complete pump (PMCA4b) has been expressed in the functionally

active state in the COS cells and in the baculovirus-infected Sf9 cells (Adamo et al., 1992b; Heim et al., 1992b). Although expression in COS cells was only 2-4 times over the background, the baculovirus system allowed a 10 to 20 fold over expression of the active pump, which was purified in an active state (Heim et al., 1992b). Latif et al. (1993) located the PMCA2 (isoform 2) in a region of the human chromosome 3 where the locus of the Von Hippel-Lindau syndrome also has been mapped. Four different genes encode plasma membrane calcium-pump named PMCA1, PMCA2, PMCA3 and PMCA4, and in addition to a variant PMCA4b.

There are 9 different primary transcripts. Some of the genes encode alternately spliced forms in the region encoding the regulatory domains of this enzymes. PMCA 1b and PMCA 4b are ubiquitous in their occurrence, PMCA 1a, PMCA 1b and PMCA 4b are expressed in excitable tissues. Whereas PMCA 1d occurs in muscle tissue, PMCA 3a in the spinal cord, and PMCA 3b in thymus, adrenal gland, spinal cord and brain. PMCA 4b which is expressed in human and bovine brain, has an insertion of an exon immediately after the sequence encoding calmodulin-binding domain resulting in lack of terminal regulatory domain (Brandt et al., 1992). A novel isoform (hPMCA3) of human erythrocyte plasma membrane Ca^{2+}-pump has been isolated. It has for 1205 a.a. with Mr 133, 930 Da and shows 75% identity (88% similarity) with previously sequenced pump isoform. Specific probes were used to detect 7.5 kb mRNA for hPMCA3 and 5.6 kb for hPMCAI. The isoform 1 (human PMCA1) gene structure has been elucidated. It has 21 exons, spanning over 100 kb, where an intron spanning over 35 kb separates 5' untranslated region from exon 1

containing translational start codon. The 5' flanking region has the putative promotor as well as numerous sp1 factor-binding sequences. TATA box is absent. This isoform of hPMCA 1 is ubiquitous in tissue distribution and is therefore considered a housekeeping gene (Hilfiker et al., 1994).

A cDNA coding for plasma membrane Ca^{2+}pump has been isolated from human teratoma library. It codes for 1220 a.a. with molecular mass, Mr 134, 683. The C-terminal of this cDNA has two calmodulin-binding domains which are enriched with glutamic acid and aspartic acid; this C-terminal plays a major role in calmodulin-mediated regulation and proteolysis (Verma et al., 1988). Rat plasma membrane Ca^{2+}-pump ATPase isoform 3 has been cloned (PMCA 3). It has 24 exons and spans over 70 kB. Splicing and polyadenylation pattern produces an alternative 4.5 kB PMCA 3 skeletal muscle mRNA that differs from 7.5 kB brain mRNA. Exon 22 is specific for skeletal muscle and significantly alters the calmodulin-binding (Burk and Shull, 1992). Neyses et al. (1985) demonstrated that Ca^{2+}-pump ATPase of cardiac plasma membrane is phosphorylated by cAMP-dependent protein kinase. Stoichiometry of kinase-mediated phosphorylation of Ca^{2+}-pump ATPase is 30%. Free Mg^{2+} ions are not essential for (formation/breakdown) turnover of calcium-pump ATPase (Verma et al., 1985).

ii. Functional properties of sarcolemmal Ca^{2+}-pump ATPase

Slow calcium-channel Ca^{2+} (influx), ATP-dependent calmodulin-sensitive Ca^{2+}-pump -Ca^{2+} efflux and Na^{+}/Ca^{2+} exchanger (Na^{+} influx/Ca^{2+} efflux) are the three vital components which mediate the calcium movement across the cell membrane. In particular Ca^{2+}-pump is

regulated by calmodulin by direct interaction and also through protein kinase activation (Carafoli, 1985). Ca^{2+} accumulation/overload in reperfused myocardium has been attributed to impaired Ca^{2+}-pump activity on the plasma membrane (Preunner, 1985). Direct correlation of plasma membrane cholesterol level coupled with impairment of Ca^{2+}-transport in myocardium was attributed to cholesterol-induced inhibition of calcium-pump ATPase (Ortega and Mas Oliva, 1986). It has been shown that there is a coordinated regulation of Ca^{2+}-binding, Ca^{2+}-stimulated ATPase and ATP hydrolysis. It requires one molecule of ATP hydrolysis for efflux of two molecule of Ca^{2+}. Further, there is a low degree of passive leakage of Ca^{2+} while ADP has also been shown to influence the Ca^{2+} transport (Dixon and Haynes, 1990).

Phosphorylation of SL calcium-pump by protein kinase C, yields two-fold acceleration of Ca^{2+} transport due to its affinity for Ca^{2+}. However, cAMP-dependent protein kinase II had no effect on phosphorylation of Ca^{2+}-pump ATPase as well as on Ca^{2+}-uptake by sarcolemmal vesicles (Ogurusu et al., 1990). Calmodulin antagonists, calmidazolium and compound 48/80 inhibited the calmodulin and phosphatidyl inositol activated calcium-pump ATPase (Pasa et al., 1992). It was hypothesized that acylphosphatase impairs the coupling of ATP hydrolysis and Ca^{2+}-stimulated ATPase-mediated Ca^{2+}-efflux (Nediani et al., 1992). In rat ventricular myocytes, the Ca^{2+} removal during relaxation is shown to be contributed by the following subcellular components and sarcolemma: SR Ca^{2+}-pump 87%, mitochondria 1.7%, Na^{+}-Ca^{2+} exchanger 8.7% and SL Ca^{2+} ATPase 2.6% (Negretti, 1993). It has been shown that when sarcolemma is enriched with cholesterol there is decrease in membrane

fluidity but Ca^{2+} channels are activated and thus there is increase in $[Ca^{2+}]_i$. Further, when sarcolemma cholesterol content is less, the membrane becomes more fluid, the calcium pump is activated, and thus more calcium is removed from cytosol resulting in a decrease in $[Ca^{2+}]_i$.

It has been shown that sarcolemma calcium-pump mediated calcium removal is not very significant during individual heartbeat (Beuckelmann et al., 1995). In ferret ventricular myocytes, by blocking the SR Ca^{2+}-pump and sodium-calcium exchange activity by adding carboxyeosin, the SL calcium-pump ATPase has been shown to be crucial in removal of $[Ca^{2+}]_i$ and for faster relaxation (Bassani et al., 1995).

B. Sarcoplasmic reticular Ca^{2+} ATPase

The sarcoplasmic reticulum (SR) Ca^{2+}-pump ATPase is a P-type ion transporting ATPase which is involved in a cyclic conformational change from phosphorylation and dephosphorylation resulting in the transport of two Ca^{2+} ions into the SR lumen per one molecule of ATP hydrolyzed. This process contributes to the relaxation of the cardiac and skeletal muscles. The SR Ca^{2+}-pump is bidirectionally oriented in the reconstituted SR vesicles. Ca^{2+} transport and coupled ATP synthesis is three times less efficient in the reconstituted vesicles compared to the native vesicles (Carafoli, 1991).

i. Structural properties of SR Ca^{2+}-pump ATPase

Active Ca^{2+} transport from the cytoplasm into SR is mediated by Ca^{2+}-pump ATPase. There are two Ca^{2+} ATPase genes: fast-twitch (SERCA 1) and cardiac (SERCA 2). The fast-twitch (SERCA 1) gene encodes two alternatively spliced transcripts which are developmentally regulated (adult SERCA 1a) and neonatal (SERCA 1b). The SERCA 2

gene also encodes two alternatively spliced transcripts, one expressed in cardiac and slow twitch muscle (SERCA 2a) and the other in non-muscle tissues (SERCA 2b). The non-muscle endoplasmic reticulum (ER) Ca^{2+}-pump ATPase isoform (SERCA 2b), is identical to the cardiac isoform (SERCA 2a) except for the replacement of the carboxyl-terminal four amino acids with an extended sequence of 49 amino acids. A third SR Ca^{2+} ATPase cDNA has been isolated from rat kidney cDNA library using oligonucleotides corresponding to part of the ATP binding site of the SR Ca^{2+} ATPases. It is of 4.5 Kb cDNA, consists of protein with Mr 109 kD, and has all of the conserved domains present in transport ATPases belonging to E_1-E_2 class. It has 77% amino acid identity with fast twitch and slow twitch/cardiac isoforms of SR Ca^{2+}-pump ATPase; also its hydropathy plus the three enzymes are identical (Zarain-Herzberg et al., 1990).

The Ca^{2+}-pump ATPase gene extends over 40 Kb, with general conservation of the intron/exon organization between SERCA 1 and SERCA 2 genes. A single transcription initiation site for the gene is located 524 base pair (bp) upstream of the translation initiation codon AUG. The promoter and 5'-flanking regions were sequenced and the "TATA" box at -28 bp, a "CCAAT" box at -83 bp and several GC rich elements were found. Deletion constructs containing various lengths of the 5'-flanking region from rabbit SERCA 2 gene upon stable transfection in C2C12 skeletal muscle cells containing a chimeric SERCA 2/CAT gene construct including 254 bp of SERCA 2 5'-flanking region showed increased transcriptional activity upon the addition of 50nM T3. There are four isoforms of plasma membrane Ca^{2+} ATPase has been cloned from rat kidney (Zarain-Herzberg et al.,

1994).

A monoclonal antibody directed against purified dog cardiac SR Ca^2-pump caused inhibition of SR Ca^{2+}-pump (ion transport) as well as ATPase activity. Octa (enthylenglycol)-monododecylether (CIZE8), a non ionic detergent, at non solubilizing concentrations interacts with SR Ca^{2+} ATPase enzyme and showed the activation to transition state in the enzyme catalysis. It should be noted that CIZE8, increase the fluidity of the lipid phase as well as the rotational diffurion of SR Ca^{2+} ATPase membrane (Anderson et al., 1983) and impaired the effect of SR monoclonal antibodies on ion transport and ATPase activities (Levitsky et al., 1987). The amino acid substitution at Gly 626 and Asp 627 prevented phosphoenzyme formation demonstrating that these residues are critical for the conformation of the catalytic domain of rabbit fast twitch SR Ca^{2+}-pump ATPase (Maruyama et al., 1989). Exposure of SR Ca^{2+} ATPase to 40% to 60% DMSO concentration resulted in random coil structure, with irreversible loss of ATPase activity. Similar loss of ATPase activity was also observed when SR Ca^{2+} ATPase was exposed to high pressure 1.5 Kbar at low temperature (2°C) (Buchet et al., 1989). Substitution of alanine for proline at Pro312→Ala; Pro 803→Ala resulted a reduction in affinity for Ca^{2+}, whereas Pro312→alanine impaired the Ca^{2+} transport ability without any effect on Ca^{2+} affinity. Based on these observations, it was concluded that the proline residue plays a vital role in structural integrity of the Ca^{2+}-binding sites in SR Ca^{2+} ATPase (Vllsen et al., 1989). Monoclonal antibodies specific to epitopes on A1 and B tryptic fragments of SR Ca^{2+} ATPase (rabbit skeletal muscle) have been raised. It has been suggested that these antibodies

may be used as a tool for analyzing the structure-function property of SR Ca^{2+} ATPase (Colyer et al., 1989).

The ATP-binding sites undergo modification resulting in half of (50%) SR Ca^{2+} ATPase inactivation and in fact myricetin, a flavanoid, has been shown to inhibit the Ca^{2+} transport property of SR Ca^{2+} pump (Thiyagarajah et al., 1991). The low affinity metal binding sites were localized to the polar head regions of the phospholipid bilayer whereas the high affinity metal binding site was localized on the stalk region of the headpiece of the Ca^{2+} ATPase and is present on the extravesicular side of phospholipid bilayer (Asturias and Blabie, 1991). Substitution of Lys 684 → Ala or histidine or glutamine impaired the Ca^{2+} transport and ATPase activity of SR Ca^{2+} ATPase (Villsen et al., 1991). It has been shown that phospholamban residues 7-16 plays a role in the regulation (stimulation) of the SR Ca^{2+} ATPase activity (Morris et al., 1991). Thapsigargin (TG), a plant extract (sequisterpene lactone), forms a complex with SR Ca^{2+} ATPase in the absence of Ca^{2+} and inhibits the steady state enzyme activity (Sagara et al., 1992). Clarke et al. (1993) showed that the transmembrane segment M_4 hydrophobic residues Ile 298 → Ile 315 are essential for Ca^{2+} binding and subsequent conformational change and translocation of Ca^{2+} by Ca^{2+} ATPase. A 14 A° resolution of SR Ca^{2+}ATPase structure has been reported. The three distinct segments in the transmembrane region are a characteristic feature of the secondary structure (Toyoshima et al., 1993). The segment Val530êArg 534 contains 5 amino acid residues vital to the sites of catalysis of SR Ca^{2+} ATPase (Lacapere et al., 1993). Cyclopiazonic acid, by enhanced stabilizing effect of dimers/small oligomers of SR Ca^{2+} ATPase protein, inhibited

the ATPase activity (Karon et al., 1994). Substitution of tyrosine residue located at TM5 uncouples the ATP hydrolysis from Ca^{2+}-transporter of SR Ca^{2+}ATPase (Anderson and Vilsen, 1995).

In megakaryoblastoid and lymphoblastoid cell lines there are two distinct forms of SR Ca^{2+}-pump ATPase with Mr 100 and 97 kD; these two forms are autophosphorylated. In other cell lines 97 kD is absent and only the 100 kD protein is present. The 100 kD form of SR Ca^{2+} pump ATPase cross-reacted with anti-PMCA antibody specific for cardiac and smooth muscle Ca^{2+} pumps. Thus presence of two distinct types SR Ca^{2+} pump ATPases may explain the differences in Ca^{2+}-handling characteristics under different [Ca^{2+}] pools in different cell types (Papp et al., 1992). Hydropathy plot analysis of amino acid sequences of SR Ca^{2+}-pump ATPase; Ca^{2+}-pump and Na^{+}/K^{+} ATPase indicated that these they have seven transmembrane domains and their C terminal are located on the cytoplasmic side of the membrane (Shull and Greeb, 1988). Korczak et al. (1988) described the structure for gene coding for rabbit fast-twitch skeletal muscle SR Ca^{2+}-pump ATPase. It has 23 exons, and transcription initiation site at +185 bp upstream for the translation initiation site. TATA box (CA-TAAA) located at positions -3O and the CCAAT sequence was found at position -78 to transcription initiation site. The region in the cytoplasmic 1A domain of phospholamban is essential for the functional association with the SR Ca^{2+}-pump ATPase (Toyofuku et al., 1994).

ii. Functional properties of sarcoplasmic reticular Ca^{2+}-pump ATPase

Contractile dysfunction in stunned myocardium is accompanied by an upregulation

of SR Ca^{2+} ATPase gene resulting in increased Ca^{2+}-pumping activity. The later increase is likely an adaptive response of the reversibly injured myocardium which may contribute to the slow recovery of contractile function (Sharma et al., 1994). It has been shown that pancreatic rough ER (endoplasmic reticulum) contains two isoforms of the SERCA 2b Ca^{2+}/Mg^{2+} ATPase whose ATP binding properties are susceptible to inactivation by vanadate but not thapsigargin (Webb and Dormer, 1995). It has been well documented that phospholamban inhibits the cardiac SR Ca^{2+} ATPase by lowering its affinity to Ca^{2+}. Both phosphorylation and nucleotide binding/hinge domains of the Ca^{2+} ATPase were shown essential for its interaction with phospholamban (Toyofuku et al., 1993).

By site directed mutagenesis in residues of cytoplasmic 1A domain of phospholamban (positively charged residues Lys3; Arg9; Arg13; Arg14 and negatively charged residues Glu2; hydrophobic residues Val4, Leu7, Ala 11, Ile 12, Ala15, Ile 18 and phosphorylation site residues Ser 16; Thr 17) resulted in loss of its inhibitory effect of phospholamban on Ca^{2+}-transport property of SERCA 2a (cardiac/slow twitch muscle Ca^{2+}-pump ATPase isozyme) expressed in HEK 293 cells (Toyofuku et al., 1994). An antibody directed against Ca^{2+}-transport ATPase of cardiac muscle SR Ca^{2+} ATPase reacted with SR Ca^{2+}-pump ATPase of slow-twitch skeletal muscle but not with fast-twitch skeletal muscle isoform of SR Ca^{2+} ATPase. Conversely antibody directed to fast-twitch skeletal muscle SR Ca^{2+} ATPase isoform did not react cardiac muscle and smooth muscle or slow-twitch skeletal muscle isoforms (Wuytack et al., 1989). Norregaard et al. (1994) demonstrated that using chimeric protein expression between SR Ca^{2+}-pump ATPase and rat kidney α_1 isoform of the

Na^+/K^+ ATPase in COS cells. Functional analysis indicates that TM_3 of the SR Ca^{2+}-pump ATPase is essential for thapsigargin sensitivity and TM_3 major binding sites for thapsigargin. The transmembrane segment M_4 plays a key role in the Ca^{2+}-transport function of the SR Ca^{2+} ATPase by participating in the binding of the Ca^{2+} and the resulting conformational changes for the translocation of the Ca^{2+} to the lumen of the membrane (Clarke et al., 1993).

C. Dynein ATPase

i. Structural properties of dynein ATPase

Dynein is made up of three globular flexible strands to a broad root-like supporting structure. Each head of the three strands has catalytic site which they interacts with microtubules in ATP concentration-sensitive manner. The force is generated when root-like structure (A) tubule interact with B tubule, leading to generation of force for biological pheriomenon called "sliding" (Johnson et al., 1986). It has been shown that headed structure dynein 22S is not required for microtubule movement indicating that multiple heads may be required for regulatory function or to generate maximal force generation for ciliary movement (Vale and Toyoshima, 1989). This protein is a structural component of intermediate chain protein of the outer row of the flagella. Any mutation in the locus affects the assembly of the entire outer row of flagella (Mitchell and Kang, 1993). Cytoplasmic dynein light gene has been cloned from chlamydomonas flagella. It is a single copy gene with one transcript detected by Northern blot. Secondary structure predicts that a amphiphilic α helix chain participates in protein-protein interaction (King and King, 1995). *Drosophila melanogaster* dynein light chain gene is an 89 amino acid (8 kD) polypeptide

mapped to 4E1-2. Mutation in the dynein light chain gene is lethal and the morphological abnormalities are similar to apoptosis (Dick et al., 1996). Based on the nucleotide sequence analysis it is clear that there are 3 different types under one family of dynein heavy chains; these are cytoplasmic dynein, axonemal dynein and DYHIB. All of these members have nucleotide binding (P loop) motif in their structure. Thus it has been hypothesized that these heavy chain genes evolved by gene duplication (Gibbons et al., 1995).

ii. Functional properties of dynein ATPase

Cytoplasmic dynein is a minus end directed, microtubule-dependent motor composed of two heavy chains (Mr 530 kD) and three intermediate chains approximately 74 kD and a family of approximately 52-61 kD light chains. Based on the two dimensional electrophoresis with peptide mapping, it was identified that the light chains are composed of two major families: a) DLC A with Mr 58,59,61 kD and DLC-B with lower Mr 52, 53, 55, 56 kD. These light chain subfamilies were tightly associated with the heavy chain tightly whereas only 74 kD intermediate polypeptides are readily extracted; both these two subunits are phosphorylated. A cDNA encoding DLC A member, has a primary structure predicted to be globular with no known proteins homology but possesses numerous potential phosphorylation sites and consensus nucleotide binding motif (Gill et al.,1994).

D. Alkaline phosphatase

i. Structural properties of alkaline phosphatase

The alkaline phosphatase [ALP; orthophosphoric-monoester phosphohydrolase; alkaline pH optimum EC. 3 .1.3.1; cDNA has been cloned and it codes for 524 residues with

17 amino acid residues in N-terminal signal sequence, hydrophilic stretch with 5 potential N-glycosylation sites and a short hydrophobic C-terminal sequence. Rat bone, liver and kidney alkaline phosphatase has 90% homology except in C-terminal region, which is a part of membrane anchoring domain. It has also been shown that it is a single copy gene (Thiede et al., 1998). Alkaline phosphatase has been considered as a cytochemical marker due to the fact that it is localized to inner and lateral membranes but not to the outer membrane surface (Gotow et al., 1985). Dephosphorylation of insulin receptor by alkaline phosphatase deactivates the receptor kinase (Czech, 1985). Formation of mature form from precursor form of alkaline phosphatase is rapid and it has been targetted to periplasm of the *E. coli* cell. (Kendall et al., 1986). Alkaline phosphatase is present in two forms namely membrane-bound alkaline phosphatase and soluble alkaline phosphatase.

ii. Functional properties of alkaline phosphatase

Membrane alkaline phosphatase contain 1 mol each of palmitate and sterate in addition to a glycerol subunit. On the other hand, glycerol subunit component is absent in the soluble alkaline phosphatase. The membrane alkaline phosphatase is attached with glycosylphospholipid as a membrane anchor and lacks the predicted carboxyl terminal peptide extension (Ogata et al., 1988). The oxidized form of alkaline phosphatase shows decreased thermal stability and biphasic heat inactivation profile. It has been suggested that Mg^{2+} binding sites in alkaline phosphatase could be the target of the ascorbate system for oxidative modifications (Mordente et al., 1988). It has been demonstrated that the alkaline phosphatase enzyme is a dimer (Olafsdttir and Chlebouski, 1989); its cDNA has been cloned.

The gene is divided into two leader exons (exon 1B and 1L) and 11 coding exons. The liver and bone specific transcriptions are regulated by their own promotors. A defect of alkaline phosphatase results in infantile hyphosphatasia, a disorder characterized by defective bone mineralization and subnormal activity of circulating alkaline phosphatase.

A monoclonal antibody raised against membrane-associated alkaline phosphatase of *Schistosoma mansoni* from splenocytes of chronically infected mice, detected this enzyme on the surface of the parasite (Pujol et al., 1990). A monoclonal antibody raised against alkaline phosphatase isolated from human osteosarcoma, localized this enzyme in human fetal bone tissue. It is highly concentrated on matrix vesicle and osteoblast plasma membrane on lateral surfaces of adjacent osteoblasts. It was concluded that alkaline phosphatase rich matrix vesicles participate in mineralization of extracellular matrix. It should be mentioned that alkaline phosphatase is a non-specific phosphomonoesterase and functions by forming a covalent phosphoseryl intermediate (E-P) (Kim and Wyckott, 1991). Placental alkaline phosphatase was demonstrated to bind IgG and participates in its internalization in IgG internalizing cell line (Hep2) (Makiya and Stigbrand, 1992). Wong and Armstrong (1992) demonstrated that the dissociation of dimeric alkaline phosphatase do not inhibit the enzyme activity but causes the alteration in its activation volume. His 412 of alkaline phosphatase plays a role in co-factor Zn^{++} binding to alkaline phosphatase and thus regulates the substrate binding as well as product release (Ma and Kantrowitz, 1994).

E. ATP diphosphohydrolase

ATP-diphosphohydrolase (ATPDase; EC3.6.1.5) hydrolyzes β and γ bonds of the

diphospho- and triphosphonucleosides. ATPDase activity was demonstrated in plant tissues, bacteria, molds, blood feeding insects, bugs, mosquitoes and also in other mammalian tissues. This enzyme has also been referred to as an "apyrase" (Beaudoin et al., 1977). Therefore it should be identified that ATP diphosphohydrolase, ATPDase and apyrase referred synonymously to the same enzyme throughout this brief review.

i. Structural properties of ATP-diphosphohydrolase

A porcine pancreatic ATPDase with a broad pH optima of 8 to 9, has been purified and characterized. Heat denaturation curves and trypsin-mediated inactivation experiments showed that there is only one enzyme involved in the hydrolysis of both ATP and ADP. Further, it has been shown that this ATPDase is a glycoprotein with Mr 65 kDa (Lebel et al., 1980). By photoaffinity labelling with 8-azido-[α-^{32}P]ATP (8-N_3-ATP), the catalytic site of (ATP/ADP binding) a 53-kDa integral membrane glycoprotein was labelled and identified to bear the catalytic domain of the ATP diphosphohydrolase (Label and Beattie, 1986).

An antiserum directed against purified bovine aortic ATPDase inhibited both the ATPase and ADPase activities. Also, both the ATPase and ADPase activities were sensitive to NaN_3. Based on these observations, it was suggested that the ecto-ATPase and ecto-ADPase are not different enzymes but expressed as one enzyme known as ATP diphosphohydrolase (Yagi et al., 1991). An ATPDase activity was shown to be present in the bovine aortic intima and media. While ATPDase present in the medial layer had more enzyme activity than intimal layer, the pH optima and electrophoretic mobility in nondenaturing gel ATPDases of bovine aortic intimal and medial origin were identical. It

was suggested that the ATPDase present in these two layers likely to a play a role in aggregation of platelets by mediating the hydrolysis of ATP released from/at the vicinity of the injured cells to ADP. Furthermore, conversion of ADP to AMP possibly reverses the platelet aggregation. These observations were interpreted as potential mechanism in controlling the thrombos formation (Cote et al., 1992).

An ATPDase with Mr 75 kDa was purified from the human umbilical vessels and shown to weakly react with the anti-bovine aortic ATPDase. Also, human umbilical vessel ATPDase is insensitive to the known ATPase inhibitors and possesses a broad substrate specificity (Yagi et al., 1992). Picher et al. (1993) has demonstrated that an ATPDase inhibited by the sodium azide (10 mM), mercuric chloride (10 μM) and gossypol (2,2'-bis [8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene]) (35 μM) is present on the bovine lung surface. Besides the ATPDases of pig pancreas (Type I) and bovine aorta (Type II), bovine lung ATPDase is referred as type III ATPDase. Also, Frassetto et al. (1993) demonstrated that an ATPDase with similar Km value for ATP and ADP hydrolysis is present in the rat blood platelets.

Sevigny et al. (1995) has purified a type-I ATPDase from the porcine pancreas zymogen granule membranes. The purified enzyme was shown to contain two distinct polypeptides with Mr 56 and 54 kD. The N-terminal sequence of Mr 56 kD peptide has substantial sequence homology with lipases whereas the amino acid sequence of the 54 kD peptide did not have sequence homology with any known proteins. Thus, it was suggested that this ATPDase is different from the other known ATPDases. The rat brain

ATPDase/apyrase activity was inhibited by the aluminum chloride, in a dose-dependent manner. This effect was reversed by the neurotoxic compound, deferoxamine (Schetinger et al.,1995). ATP diphosphohydrolase with Mr 63 and 55 kD was purified from the tegumental membranes of Schistosoma mansoni. However, the 63 kD band showed immunocross reactivity with anti-potato apyrase antibody and anti-potato apyrase antibody immunoprecipitated both ATPase and ATPDase activities from the tegmental membranes (Vasconcelos et al., 1996). In the rat heart sarcolemma, the hydrolysis of ATP and ADP were mediated by single enzyme referred to as ATP-diphosphohydrolase or apyrase. It was suggested that ATPDase acting in concert with 5'-nucleotidase likely to be involved in the dephosphorylation of nucleotides in the interstitial region (Espinosa et al., 1996).

ii. Functional properties of ATP-diphosphohydrolase

Based on the regulatory studies performed on ATPDase of the human placental and rat renal microvilli, it was suggested that there is an activating factor in the cytosol and an inhibitory factor in the membrane associated proteins present on the surface of placental microsomes (Valenzuela et al., 1996). In rat xenograft aorta model, continuous administration of soluble apyrases/ATPDase conferred a sustenance of the grated tissue. (Koyamada et al., 1996). The amino acid sequence of the vascular ATPDase has been shown to have maximum sequence homology with CD 39. Tissue distribution analysis showed that the presence of multiple transcripts in the human placenta, lung, skeletal muscle, kidney, heart and brain. It has been suggested that overexpression of the functionally active enzyme is likely to restore the inhibitory effect on the platelet activation (Kaczmarek et al.,

1996). An ecto-ATPDase (glycoprotein) with Mr 80kD hydrolyzing both the nucleotide triphosphates and nucleotide diphosphates was purified and characterized from the chicken oviductal vesiculosomes. The uniqueness of this ecto-ATPDase is that it has the highest ATPase activity reported to date (Strobel et al., 1996). Bovine aortic smooth muscle ATPDase has been shown to hydrolyze the P_2 purinergic agonist (Picher et al., 1996). An ATPDase was purified from the "right side out" rat renal microvillar membrane; this enzyme was also detected on the surface of the microvilli. The ecto nature and existence of the ATPDase in the vascular endothelium was identified by perfusion studies in the isolated rat kidney with ATP and ADP both in the presence and absence of various inhibitors and also with the anti-apyrase antibodies (Sandovel et al., 1996).

The human placental ATP diphosphohydrolase (ATPDase) was localized on the plasma membranes of placenta. Deglycosylation with N-glycosidase F yielded a 57.5 kD ploypeptide protein from its original Mr 82 kDa. Deglycosylated molecule has retained ATPDase activity in solution as well as in ATPDase activity staining under native gel electrophoresis (Christoforidis et al., 1996). In rat renal glomerular ischemia-reperfusion injury model, 30 min ischemia-reperfusion resulted in a loss of the glomerular ATPDase activity. However, prior exposure to the soluble complement receptor type 1 (sCR1) or antioxidants conferred a protective effect on the glomerular ATPDase activity (Candinas et al., 1996).

An ATPDase with Mr 78 kD was purified and characterized from the bovine lung. This enzyme has been shown to possess immunocrossreactivity with an antibody directed

against bovine pancreas ATPDase, ATPDase in the lung airway epithelial cells, airway smooth muscle cell ATPDase, and in vascular smooth muscle cells. It was suggested that the bovine lung ATPDase may play a pivotal role in the smooth muscle tone, surfactant secretion, platelet secretion as well as in the inflammatory response (Sevigny et al., 1997).

The bovine aortic endothelial ATPDase activity was enhanced by monounsaturated fatty acid (oleic acid) supplementation in the culture. Whereas the polyunsaturated fatty acids exerted an inhibitory effect on ATPDase activity. It suggested that the observed modulation of ATPDase activity could be due to the changes in the membrane phospholipid composition of endothelial cell membranes (Robson et al., 1997). Rat platelet ecto-ATP diphosphohydrolase/apyrase/ATPDase exposed to the free radicle generating system exhibited a marked reduction in the activity. Whereas, glutathione (GSH) and cysteine conferred protection against the free radical-induced loss of ATPDase activity. Based on this observation, it was suggested that the loss of ATPDase activity may possibly be due to the modification of the amino acid residues present in the catalytic domain (Frassetto et al., 1997). Based on several lines of evidence (maximal ATPase activity at different pH; sensitivity to different inhibitors; metal ion requirement) that the ATP hydrolytic property present on the surface of the *Leishmania tropica* has been identified as a Mg^{2+}-dependent ecto-ATPase activity but not an ATPDase (Meyer Fernandes et al., 1997).

The human umbilical vein endothelial cell (HUVEC) ADPase has been classified as an E-type ATP-diphosphohydrolase and also referred to as an CD39. COS cells transfected with the CD39 cDNA conferred an inhibitory effect on ADP-induced platelet aggregation

in the platelet-rich plasma. Thus it was suggested that CD39/ecto-ADPase may act as a thrombos regulator exhibiting a potent inhibitory effect on platelet aggregation in HUVEC cells (Marcus et al., 1997). From the bovine aortic medial cells an ATP diphosphohydrolase, with a Mr 78 kD has been purified and characterized. ATP-binding site was identified by FSBA labelling method. This enzyme showed immunocrossreactivity with antibodies directed against the N-terminal region of the pig pancreas ATPDase (54 kD) (Sevigny et al., 1997). Beaudoin et al. (1997) has purified and characterized two ATP diphosphohydrolase isoforms from the bovine heart ventricle. These two isoforms of the bovine cardiac ATPDases possesses an immunocrossreactivity with the antibodies directed against amino terminal sequence of the porcine pancreatic ATPDases. These new isoforms were suggested to play a crucial role in conferring the protective role against the lytic effect of the extracellular ATP. It has been shown that the progression of vascular injury is result of the loss of vascular ATPDase activity (Robson et al., 1997).

F. The cell membrane Na^+/K^+ ATPase

The major ion motive ATPase is the ouabain-inhibitable sodium-pump, a membrane-bound enzyme that couples the free energy contained within the ATP molecule to the translocation of Na^+ and K^+ across the plasma membrane.

i. Structural properties of Na^+/K^+ ATPase

In eukaryotic cell the free energy released upon ATP breakdown is utilized for the transport of three Na^+ (efflux) and two K^+ (influx) across the plasma membrane by Na^+/K^+ ATPase or sodium-pump. The sodium-pump is made up α- and β-subunits and undergoes

E_1-E_2 transition, a characteristic feature of a P-type ATPase. Three α-subunit isoforms (α_1, α_2 and α_3) and two β-subunit isoforms (β_1 and β_2) were isolated. It was suggested that isoforms confer evolutionary advantage and regulation of gene expression during development in a tissue-specific manner (Takeyasu et al., 1988). The differential expression of Na^+/K^+ ATPase in different cells or tissues is due to its structural heterogeneity (Horisberger et al., 1991). By X-ray microdialysis of frozen cryosections, the Na^+/K^+ pump is proposed to be present in the apical and basal cell membrane (Gupta and Hall, 1993). A monoclonal antibody anti-BSP-3 directed against brain cell surface protein recognized mouse sodium-pump (E.C 3.6.1.3). Also, this antibody immunoprecipitates ATPase activity in microsomal fraction of mouse kidney. Basolateral cell surface of polarized cells was stained by BSP-3 antibody (Gorvell et al., 1983). The α-subunit (MW 84 kD → 120 kD) is considered to play a vital role in the catalytic activity of sodium-pump (Kaeakami et al., 1985). Sheep kidney sodium-pump β-subunit (MW 55 kD) cDNA was cloned. It has been shown that structurally β-subunit amino acid sequence is similar to Kdp c subunit of Na^+/K^+ ATPase subunit of *E. coli* (Shull et al., 1986). The β-subunit of the sodium-pump has been shown to play a critical role in cellular resistance to cardiac glycosides (Mercer et al., 1986). By decreasing the α-helix conformation of plasma membrane proteins, the high $[Ca^{2+}]_i$ inhibited the sodium pump (Vrbjar et al., 1986). Expression of entire mouse cDNA coding for α-subunit conferred ouabain resistance in monkey CV-1 cells; upon deletion of the C-terminal of the α-subunit cDNA resulted in impairing this property (Kent et al., 1987).

ii. Functional properties of Na^+/K^+ ATPase

Amino acid substitution Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn- ò Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp, in the N-terminal, extracellular domain of the α in HeLa cells conferred ouabain resistance (Price and Lingrel, 1988). The three alpha subunit isoform gene structures have been characterized. All three isoforms have similar exon-intron structure (Broude et al., 1989). β-subunit upregulation modulates the number of potassium-pump formation and translocation to the membrane surface. It also regulates the α-β heterodimer formation (McDonough et al., 1990). Vascular smooth muscle sodium-pump α-subunit isoform formation is regulated by alternative splicing of single transcript (Medford et al., 1991). 8-thio ATP (CNS-8-ATP) is bound to ATP binding site in the sodium-pump and mediates the inhibitory effect. Addition of ATP removes the inhibition. The activity of inactivated enzyme is restored by incubating with DTT (Scheiner-Bobis et al., 1992). Substitution of amino acids α_1 C113→Y and α_1 C113→F of the sodium-pump renders ouabain resistance compared to the wild type (Cannessa et al., 1992). It has been shown that sodium-pump activity is regulated in part by L cell liver fatty acid binding protein by increasing the plasma membrane fluidity (Incerpi et al., 1992). Substitution of amino acid in α_1-subunit ectodomain H_3-H_4, Y317C, causes increased ouabain resistance in Madin-Darby canine kidney cell line by affecting the ouabain binding to α-subunit (Canessa et al., 1993). Amino acid residues 496-HLLVMKGAPER-506, forms the FITC binding domain in the catalytic α-subunit of the sodium-pump. 494-PRHLL-498 is the most critical sequence for nucleoside binding (Malik et al., 1993). Transfection of HeLa cells with double

mutants generated by the amino acid substitutions at Asp111 and Arg122 in the H_1-H_2 extracellular domain of sheep sodium-pump α-subunit, rendered ouabain resistance to the cells. It was suggested that H_1-H_2 extracellular domain of sodium-pump α-subunit possesses the functional unit of the sodium-pump (Price et al., 1990).

The yeast sodium-pump is made up of α- (catalytic) and β- (structural) and putative γ-subunit. Presence of α,β-subunit complex alone confers the ouabain-inhibitable sodium-pump activity where as the γ-subunit is redundant (Scheiner and Bobis-Farley, 1994). The Cys104 in H_1 transmembrane domain of α-subunit of sodium-pump possesses the cardiac glycoside binding site (Antolovic et al., 1995). The α-subunit hydrophobic domain H_1 and H_3 serves as signal-anchor type II and H_2-H_4 confers "halt" transfer signal, whereas membrane insertion property of sodium-pump is conferred by amino-terminal possessing signal/anchor type II/halt transfer sequences (Xie and Morimoto, 1995). The region corresponding to Gly554→ Pro785 in the cytoplasmic domain of α-subunit is essential for complex formation (Koster et al., 1995). Substitution of amino acid at D369→N porcine kidney sodium-pump α-subunit caused an 18-fold increase in ATP binding by net reduction in negative charge in the phosphorylation site D369→N (Pederson et al., 1996). Based on hydropathy plot, different transmembrane domains, varying from six (Kawakami et al., 1985); seven (Orchininikove et al., 1987) and eight (Shull et al., 1985) have been proposed for the α-subunit. The H_1-H_2 and H_3-H_4 domains were orienting to the extracellular face and FITC [ATP] binding site, facing the cytoplasm (Horisberger et al., 1991). α-subunit N-terminus and C-terminus in the α-subunit are present in the cytoplasm; N-terminus is the

most divergent where the H_1-H_2; H_3-H_4 domains are shown to participate in ouabain binding. Amino acid residues Asp 369 and Lys 501 were labelled by FITC. Cys356, Asp 710 and Lys 719 were identified as ATP binding region of α-subunit (catalytic subunit) (Jorgenssen and Anderson, 1988; Swaedner, 1989; Shull et al., 1985).

The α-subunit isoform distribution is well conserved across the species (see review Swaedner, 1989). The β-subunit has been shown to mediate the membrane insertion and its transmembrane domain is well conserved across the species. The extracellular domain of different isoforms of β-subunit has different glycosylation sites. Six cysteine residues are critical for the disulfide bridge formation in all beta isoforms (Kirley, 1989). It has been conclusively demonstrated that the stoichiometry of sodium-pump is 3 Na^+(efflux):2 K^+ (influx) and is similar in epithelial and excitable cells (Swaedner, 1985).

G. Actomyosin ATPase

Myosin is an ATPase molecule which forms an active component of the actomyosin filament. The actomyosin complex generates contraction is in the presence of ATP, K^+ and Mg^{2+}.

i. Structural properties of actomyosin ATPase

The actin-activated myosin ATPase is inhibited by fodrin isolated from brain tissue; the fodrin-induced inhibition of actomyosin ATPase occurs in the absence of Ca^{2+} (Wagner, 1984). 6-Tridecylresorcyclic acid also inhibits the actomyosin ATPase and contraction of contractile protein in a dose-dependent manner (Kobayashi et al., 1984). Antibodies M 2.2, M-15 and M-17 uncoupled the myosin ATPase activity from gel-sol contraction; this

inhibition has been attributed to the binding of antibodies to myosin II heads (Kiehardt and Pollard, 1984). Caldesmon (150 kD), which inhibits tropomyosin, was found to enhance the actomyosin ATPase activity in a Ca^{2+}-independent fashion and is considered to play a regulatory role in inhibiting the reaction (Sobue et al., 1985). Based on immunological evidence, caldesmon has been shown to be present on the thin filament (Marston and Lehman, 1985). The troponin-tropomyosin complex is also shown to inhibit the kinetic step in the actomysosin ATPase cycle and is thus known to regulate the actomyosin ATPase activity (King and Greene, 1985). C-proteins in thick filaments of the striated muscle have been shown to enhance the formation of stable aggregates among actin myosin filaments and thereby activate the myosin ATPase activity (Hartzell et al., 1985). Troponin C (89-100) and troponin I interact with each other (Dalgarno et al., 1982) and this interaction is required for the Ca^{2+}-sensitivity of actomyosin ATPase (Syska et al., 1976). It has been shown that the Ca^{2+}-binding at the low affinity site in troponin C triggers the alteration in its conformation thereby facilitating the contact between troponin C and troponin I (Grabarek et al., 1986). Tropomyosin-actin phosphorylated form is inactivated by caldesmon binding to actomyosin complex and Ca^{2+} calmodulin complex reverses this inhibition without the removal of caldesmon from the complex. Furthermore, it has also been shown that caldesmon phosphorylation occurs during the ATPase cycle (Horiuchi et al., 1986).

Stoichiometry of one mol of tropomyosin:to seven mol actin monomer is required to maximize the myosin ATPase activity and Ca^{2+}-sensitivity (Miyata and Chacko, 1986). A chymotrypsin-digested caldesmon product, a 40 kD fragment, preserves the functional

properties of the undigested caldesmon molecule (Szpacenko and Dabrowska, 1986). Caldesmon purified from bovine aortic smooth muscles has been implicated in enhancing cross-bridge affinity while preserving functional identity, i.e. inhibition of actomyosin ATPase activity (Lash et al., 1986). The low affinity Ca^{2+}-specific binding site (Site II) of skeletal muscle troponin C of rabbit comprises the residues between 52-62 amino acids; this site has been proposed to be the site for troponin I interaction with troponin C (Malik et al., 1987).

Amino acid sequence comparison of tropomyosin-binding subunit of troponin T from chicken, bovine and rabbit cardiac tissue showed highest homology of 13 amino acid residues in the N-terminal. This component of thin filament regulatory complex provides Ca^{2+}-sensitivity for contraction and actomyosin ATPase activity (Leszyk et al., 1989). It has been demonstrated that troponin T binds to N-terminus region of tropomyosin (Hitchock-Degrgori and Heald, 1987). The caldesmon-induced impairment of actomyosin ATPase activity is reversed by the calcium-calmodulin (1:8 molar ratio) in the presence of Ca^{2+}. The reversal of inhibition by caldesmon does not necessitate the release of caldesmon from actin (Chacko et al., 1987). It has been demonstrated that caldesmon regulates its biological function by Ca^{2+}/calmodulin-dependent autophosphorylation, and also by phosphorylating synapsin-I in Ca^{2+}/calmodulin-dependent manner (Scott Woo and Walsh, 1988). Bovine cardiac troponin I and rabbit cardiac troponin I amino acid sequence have been shown to have maximum sequence homology. 12/26 at N-terminal and 97% homology rest of the molecule (Leszyk et al., 1988). Fetal smooth muscle myosin light chain is

phosphorylated by protein kinase C on serine or threonine which in turn stimulates actomyosin ATPase activity in fetal myosin. On the contrary, adult smooth muscle myosin light chain is phosphorylated by Ca^{2+}/calmodulin-dependent myosin light chain kinase which in turn stimulates actomyosin ATPase activity (De Laneolle and Nishikawa, 1988). Utilizing antibodies directed against caldesmon (aortic native smooth muscle thin filaments), it was demonstrated that caldesmon is a Ca^{2+}-dependent inhibiting component present in vascular smooth muscle thin filaments (Marston et al., 1988). By isoelectric focusing two variants of mammalian vascular smooth muscle myosin light chain has been identified. The light chain variants have pI 4.13 and pI 4.19 with obvious difference in peptide composition and varied content in different tissues (Helper et al., 1988).

Troponin I is the inhibitory subunit present in actomyosin ATPase of myosin thin filament. The consensus sequence of actin-binding domain of troponin I is D-L-R-G-K-F-X-R*-P-X-L-R*-R-V, where R is for Arg/trimethyl lysine and X = any amino acid residue. The high sequence identity in the troponin T in C-terminus of troponin I has been implicated in protein-protein interaction (Kobayashi et al., 1989). Caldesmon binds to NH_2-terminal on actin thereby competes with myosin-ATP and is thus considered to play a role in regulation of actomyosin ATPase (Adams et al., 1990). Calponin present in chicken gizzard smooth muscle has been shown to inhibit the actin-activated Mg^{2+} ATPase activity of smooth muscle myosin possibly by regulating the actin-myosin interaction and thus the contractile state of smooth muscle. Ca^{2+}-dependent phosphorylation of calponin is suggested to regulate its function (Winder and Walsh, 1990). Troponin I, a subunit in troponin complex present

in striated muscle has been shown to inhibit the actomyosin ATPase activity. Troponin I has been cloned from rat cardiac specific isoform and has the highest amino acid sequence homology with bovine and rabbit cardiac troponin I. Expression of this isoform is high in adult cardiac tissue compared to the embryonic stage and it is confined to heart tissue because it is not expressed in skeletal muscle at any developmental stage (Martin and Orlowski, 1991).

The 38 kD domain of caldesmon and its interaction with tropomyosin-actin affects the binding of heavy meromyosin to actin and also the ATPase cycle. Thus it has been suggested that the caldesmon 38 kD fragment (Chymotrypsin fragment) plays a role in the regulation of actomyosin ATPase and the contraction of the smooth muscle. (Horiuchi et al., 1991). The amino acid residue arginine13-arginine16 is required for the actin-activated ATPase in smooth muscle myosin; this is mediated by the phosphorylation of serine 19 of myosin light chain (Ikebe and Morita, 1991). Chymotrypsin-treated caldesmon yields a 7.3 kD spanning Leu597→Phe665. This fragment binds to actin in 1:1 ratio to form a complex. Based on this observation it was suggested that this is the smallest region regulating the interaction between caldesmon with calmodulin and actin (Chalovich et al., 1992). It was suggested that the N-terminal segment is catalytically important to actomyosin ATPase activity (Cheung and Reisler, 1992). Papain digested product of porcine aorta smooth muscle myosin subfragment heavy chain C-terminal a ~ 2 kDa peptide (S-1, 835→846) has been shown to regulate smooth muscle actomyosin ATPase activity by phosphorylation of the regulatory light chain (Katoh and Morita, 1993). Fluoroaluminate

and fluoroberyllate stimulate the phosphate analogues by binding at the active site where the γ phosphate of ATP binds (Henry et al., 1993).

N-helix conformation of troponin C is required for Ca^{2+}-binding to the high affinity sites and also for its stability in view of Ca^{2+}-switching mechanism (Smith et al., 1994). Asn 675→Trp722 region in the carboxy-terminal of 10 KDa domain of caldesmon has been shown to play a regulatory role (Mezgueldi et al., 1994). Rabbit skeletal muscle myosin subfragment I binds to the actin (338-348) hydrophilic segment with high affinity in the absence of Mg^{2+} ATP (Labbe et al., 1994). The N-terminal of Tn I domain extension is essential for differential regulation of acidic pH on Ca^{2+} activation in cardiac and skeletal myofilaments (Guo et al., 1994). The transcription unit of troponin I slow muscle specific isoform of rat has been shown to have 10.5 kB with 9 exons (4 bp→330 bp) and a 3.3 kB intron separates the 5' untranslated regions from protein-coding exons. Exons/intron map of quail Troponin I (fast) and rat troponin I (slow) are similar except that the rat gene contains an additional exon in 5' untranslated region (Bannergee and Buananno, 1994).

ii. Functional properties of actomyosin ATPase

The C-terminal domain of the myosin light chain is essential for transmission of conformational change in regulatory light chain by ser19 phosphorylation of myosin heavy chain (Ikebe et al., 1994). It has been shown that TnC binding with Ca^{2+} releases the Troponin I induced by ATPase and in turn activates the ATPase by interaction with Troponin I (Zhang et al., 1995). The proposed multifunctional domain 145→163 region in the N-terminal of calponin plays a vital role in interaction of calponin-F actin, thus

enhancing the inhibition of actomyosin ATPase. This mechanism has been demonstrated to play a positive regulatory role in smooth muscle calponin (Mezgueldi et al., 1995). Adrenaline-induced Ca^{2+}-sensitivity of myofibrillar actomyosin Mg^{2+} ATPase depends on second phosphorylation at ser 23 of cardiac troponin I (Quirk et al., 1995). The region corresponding to the amino acid residues 728→756 and 718→727 in caldesmon is required for its interaction with tropomyosin and inhibition of tropomyosin-mediated actomyosin ATPase activity (Horiuchi et al., 1995). The VKYAEK of calponin is critical for ATPase inhibition and it represents part of the actin-binding domain (el Mezgueldi et al., 1996). Troponin I interaction with Troponin C takes place at the N-terminal Glu 60 and/or Glu 61 and acidic residues in linker region 84-94 which links – and C-terminal of troponin C (Kobayashi et al., 1996).

5. V-type ATPase

A. H^+ ATPase/vacuole ATPase

It has been suggested that vacuolar membrane ATPase (VMA) plays a role in ionic homeostasis in cytosol of the intracellular vacuoles.

i. Structural properties of H^+ ATPase/vacuole ATPase

This enzyme, which is referred to as H^+ translocating ATPase/H^+ ATPase/V type ATPase, was purified as a single complex (Kanazawa et al., 1983). H^+ ATPase gene of *Neurospora crassa* has been cloned and characterized. Its amino acid sequence has 75% homology with *Saccharomyces cerevisiae* and plasma membrane H^+ ATPase. Considerable homology was observed with Na^+/K^+ ATPase and Ca^{2+} ATPase in certain regions and it was

hypothesized that phosphorylatable ion transporting ATPases function in a similar energy transduction mode (Addison, 1986). Vacuolar ATPase from *Neurospora crassa* is made up of two polypeptides with Mr 70 kD and 60 kD. VMA-1 gene codes for a polypeptide 70 kD and possesses the ATP hydrolytic property (Bowman et al., 1988) whereas VMA-2 gene codes for polypeptide 60 kDa. The sequence analysis suggests that it may be functionally similar to α-subunit of F_1F_0 ATPases (Bowman et al., 1988). The N-terminal amino acid residues 28→60 are required for the localization of H^+ATPase to the membrane. The C-terminal 11 amino acid residue constitutes the regulatory domain of H^+ ATPase (yeast) (Portillo et al., 1989). The yeast vacuolar membrane protein (H^+ ATPase) has been purified and the corresponding gene structure is known. H^+ ATPase is made up of 3 subunits (MW 67 kDa, MW 57 kD, and MW 20 kD). Subunit A possesses the catalytic property, subunit B has an as yet unidentified function and subunit C acts as a channel translocater. Thus it has been suggested that VMA plays a role in ionic homeostasis in cytosol (Anraku et al., 1989).

The yeast H^+ ATPase is inhibited by 0.1 M KNO_3 in the presence of 5 mM Mg^{2+} ATP. Inhibition of ATPase activity and disassembly of H^+ ATPase by < 200 mM KNO_3 suggests that it is a conformation specific reaction (Kane et al., 1989). The neurospora plasma membrane H^+ ATPase was inhibited by N-ethyl maleimide (NEM); this inhibition is by a specific interaction of NEM with cys 532 of H^+ ATPase (Pardo and Slayman, 1989). It has been shown that the NEM interaction with H^+ ATPase results the inhibition of phosphoenzyme formation by H^+ ATPase. Cys 532 residue is directly modified by NEM

interaction (Chang and Slayman, 1990). Substitution of amino acids Asp378→Glu or Ser or Asn and Lys 379→Gln abrogates the biogenesis of yeast plasma membrane H^+ ATPase. Substitution of Lys 379→Arg caused 6-fold decrease in ATPase activity. Inactivation of H^+ ATPase was observed when substitutions were made in Thr 380, Thr 382 or Thr 384 by Ala. Thus, it is evident that amino acid residues at 379→384 plays a role in ATP hydrolysis (Rao and Slayman, 1992).

ii. Functional properties of H^+ ATPase/vacuole ATPase

The H^+ ATPase present in the coated vesicle serves to confer the acidic environment in the endosomes as well as in the intracellular compartments required for receptor recycling and intracellular membrane transport. Structural analysis indicates coated vesicle H^+ ATPase are structurally similar to F-type ATPase (Forgae, 1992). The sequence comparison of cloned V-type and F-type ATPase reveal that these two families must have evolved from common ancestral genes. Presence of peripheral catalytic domains and hydropathic membrane domains are unique to these two families of ATPases (Nelson, 1992). A classical overview on how the mechanism of evolution bestowed the two progenies (V-type/F-type) from a founder has been presented in a review by (Kibak et al., 1992). Formation of catalytic domain and noncatalytic domain of F-type/V-type ATPases are evolved by gene duplication. Isoforms formation for V-type ATPase/catalytic subunit may imply that their functional properties are destined in a tissue and organelle specific manner (Gogarten et al., 1992). V-type ATPases are predominantly found in chromaffin granules and clathrin coated vesicles. The subunit stoichiometry is in the order of 3A:3B:1C:1D:1E:6C (proteolipids):

1AC115 and AC39. The catalytic and membrane domain are interdependent for its (H^+ATPase) optimal activity (Nelson, 1992). The functional properties unique to coated vesicle V ATPases include: receptor-mediated endocytosis, ligand receptor dissociation and receptor recycling in acidic intracellular environment (Nelson, 1992).

H^+ ATPase is made up of nine subunits with MW 750 kD organized in two structural entities namely peripheral V_1 domain with nucleotide binding domain (Mr 500 kD) and integral V_o domain (Mr 250 kD) involved in proton conduction process (Forgac, 1992). Chicken osteoclast membranes contain a typical H^+ ATPase inhibited by V-type inhibitor [NEM; Bafiliomycin A_1] and P-type ATPase inhibitor, vanadate. The osteoclast H^+ ATPase also possesses the isoform of subunit A with MW 63 kD and subunit B is present in two different types 57-60 kD (Chatterjee et al., 1992). It has been shown that the nephros (Crustacean: *Nephros norvegius*) proteolipids play a role in membrane transport functions. 16 kD proteolipid and VMA-3 gene coding for *S. cerevisiae* integral membrane component of V-type ATPase share the structural similarities at the regions of protein interaction (Harrison et al., 1994). The cytolytic property of $CD8^+$ in cytotoxic T lymphocytes is directly correlated for its optimal function with acidification by vacuolar type H^+ ATPase (Kataoka et al., 1994). VMA-21 strain is a mutant, lacking V-type functions. The dilysine motif present in the C-terminal abrogates the assembly of the integral membrane subunit of V-type ATPase (Hill and Stevens, 1994).

2-azido[^{32}P]ATP labelling was observed in 12 kD polypeptide spanning 511 amino acids composed of Cys 532 and also a 3 kDa fragment with residue 233 encompassing

glycine rich loop as well Cys 254 (Zhang et al., 1995). The 16 kD proteolipid of V_o domain of H^+ ATPase forms the pore structure for proton translocation (Jones et al., 1995). In H^+ ATPase of *S. cerevisiae* β-subunit (VMA-2p) substitution of amino acids R381→S and Y352→S resulted in impairment of the ATPase activity as well as proton transport (Liu et al., 1996). Substitutions of amino acids at S811P and E740D in VMA-1 gene resulted in normal assembly but the ATPase activity was impaired. Y797H mutant resulted in partial defect in growth and ATPase activity with the deletion of two peripheral subunits of H^+ ATPase (Liu and Kane, 1996).

Vacuolar H^+ ATPases play a major role in generating proton-motive force across the membranes of organelles connected with vacuolar systems of eukaryotic cells. A gene encoding N,N-dicyclohexylcarbodiimide (DCCD) binding protein proteolipid of the H^+ ATPase of bovine chromatin granules had been cloned with Mr~16 kD. Hydropathic plot analysis showed glycine residue present in transmembrane domain is the DCCD binding site. Sequence analysis indicates that proteolipids of vacuolar H^+ ATPases were evolved in parallel with eubacterial type from a common ancestral gene but eventually these underwent a gene duplication process (Mandel et al., 1988). Vacuolar proton (H^+) ATPase is inactivated upon exposure to cold duly releasing 5 polypeptides with Mr 72, 57, 41, 34, 33 kD. Polypeptides 72, 57, 34 kD were identified as subunits of vacuolar H^+ ATPases by antibody crossreactivity (Moriyama and Nelson, 1989). Wang et al. (1989) have cloned a cDNA encoding an accessory polypeptide of H^+ ATPase from bovine adrenal medulla cDNA library. It codes for a protein of Mr 31 kD. Its electrophoretic mobility in SDS-PAGE is

similar to that of its subunit of purified H^+ ATPase.

Tissue distribution analysis indicates a single transcript of 1.6 kB size in bovine adrenal medulla, whereas in lung, liver and kidney, an additional 1.7 kB transcript was detected. V-type ATPases are ATP-dependent proton pumps responsible for acidification of intracellular compartments in eukaryotic cells. They play a role in receptor-mediated endocytosis, transcellular membrane traffic, macromolecular processing and degradation and coupled transport. It has V_1-complex for ATP hydrolysis and integral V_0-domain responsible for proton translocation. Regulation of vacuolar acidification is critical to its role in membrane traffic and other cellular processes (Rodman et al., 1994).

B. H^+/K^+ ATPase

i. Structural properties of H^+/K^+ ATPase

The activity of H^+/K^+ ATPase depends on H^+ and K^+ and this enzyme was suggested to be involved in gastric HCl secretion. The enzyme has two ATP-binding sites whereas Mg^{2+} as a cofactor to catalyze the exchange of H^+ for K^+ with ATP hydrolysis. Without ATP, this ATPase functions as a passive exchanger for K^+ at a lower rate (Sourmon and Lewin, 1986). The N ethyoxy-1-ethyl-substituted benzimidazoles inhibited rat H^+/K^+ ATPase activity (Sih et al., 1991). The proton pump or H^+/K^+ ATPase (E.C.3.6.1.3) is made up of an α-subunit which is the catalytic portion of the enzyme, β-subunit 60-90 kD protein (Sachs et al., 1989). A monoclonal antibody raised against canine gastric membrane antigen, recognized luminal epitopes present on the core protein of beta-subunit (Jones et al., 1991). It has been shown that the β-subunit of Na^+/K^+ ATPase and H^+/K^+ ATPase are structurally

similar (Eakle et al., 1992). Omaperrazole has been shown to inhibit the H^+/K^+ ATPase in gastric glands in a specific manner (Fryklund et al., 1988). The H^+/K^+ ATPase of toad (*Bufo marinus* - amphibian) collecting tubule has been shown to regulate H^+ and K^+ homeostasis by mediating the transport of these ions across the urinary epithelia and thereby regulating urinary excretion (Jaisser et al., 1993). Topological analysis of gastric H^+/K^+ ATPase indicates, the presence of several membrane segments; fourth and eighth are the membrane spanning segments. These findings are contrary to the earlier observations that membrane segments M5, M6 and M7 of gastric H^+/K^+ ATPase were determined differently and that M1, 2, 3, 4 and 8, the M9, M10 are the membrane spanning segment (Bambery and Sachs, 1994).

ii. Functional properties of H^+/K^+ ATPase

Heterologous expression of the β-subunit of H^+/K^+ ATPase with α-subunit in *E. coli* led to the assembly of the two subunits for the enzyme to serve as an ion pump. Ouabain binding to this active ion pump has also been shown (Eake et al., 1992). An antibody directed against lysosomal membrane showed reactivity to 100 kD protein in the acidic vacuolar compartment and H^+/K^+ ATPase from the pig gastric mucosa. This antigen is present on the plasma membranes in the liver as well as in vesicles near the sinusoidal surface of hepatocytes, (putative endosomes), the Golgi complex adjacent vesicles and vacuoles and pericanalicular dense bodies (Reggio et al., 1984). A monoclonal antibody HK_{4001} directed against hog gastric H^+/K^+ ATPase inhibited its activity at a molar ratio of 1:2 (antibody/catalytic subunit) at pH 7.8. HK_{4001} inhibited gastric H^+/K^+ ATPase from rabbit

and rat but did not crossreact with any cation transport ATPase (Na^+/K^+ ATPase or Ca^{2+} ATPase) or H^+ ATPase in the multivesicular body. It was suggested that HK_{4001} recognizes the highly specific site on the cytosolic surface of H^+/K^+ ATPase. It has been suggested that the functional unit of H^+/K^+ ATPase is a dimer or tetramer of the catalytic unit (Asano et al., 1989).

Glycosylation on the β-subunit of the gastric H^+/K^+ ATPase may protect the enzyme from acidic environment in the stomach (Chow and Forte, 1995). Utilizing ATP_tAL_t gene as probe from rat colon library, a cDNA coding for 1O36 amino acids with Mr 114, 842 kD has been cloned and characterized. Protein/amino acid sequences exhibit a 63% a.a. homology with gastric H^+/K^+ ATPase subunit and a 63% identity with Na^+/K^+ ATPase subunit isoforms. 4.3 kb mRNA had a higher expression at the distal end of the colon but much lower expression in the proximal colon, kidney, uterus, heart and fore stomach was evident; higher level of expression in the distal colon suggests that it is a distal colon H^+/K^+ ATPase. Furthermore its expression in kidney indicates that it may play a role in K^+ absorption and H^+ secretion in the distal nephron (Crowson and Shull, 1992). The catalytic subunit of the H^+/K^+-transporting ATPase has 62% identity with catalytic subunit of the Na^+/K^+-transporting ATPase. Deglycosylated form of the subunit purified from rat H^+/K^+ ATPase has a Mr 35 kD. Upon fragmentation of H^+/K^+ ATPase with protease V_8, it gives sequences homologous to both subunits 1 and 2 of Na^+/K^+ ATPase cDNA sequence of subunits of H^+/K^+-transporting ATPase with open reading frame (54↘926) encodes 291 a.a. (Mr 33,32O Da) and shows 31% homology with subunit 1 of Na^+/K^+ ATPase and 44%

homology with subunit 2 of Na^+/K^+ ATPase protein. H^+/K^+ ATPase cDNA detects a 1.3 to 1.5 kb in hog stomach (Reuben et al., 1990).

It has been shown that omeprazole inhibits the gastric proton pump but it has no effect on the H^+/K^+ ATPase gene expression despite adaptive changes in the stomach endocrine system (Loventzon et al., 1997). H^+/K^+ ATPase is present in the cytoplasmic membrane of apical surface of gastric parietal cell and tubulovesicles. Upon stimulation, the ATP-driven H^+/K^+ ATPase is redistributed and thus there is an enhanced HCl secretion. KCl cotransport is also enhanced and operates in concert with ATP-driven H^+/K^+ exchange pump. It has been demonstrated that both systems operate in concert to confer HCl transport in gastric parietal cell (Forte and Lee, 1993). Gastric microsomal membrane organization and complete structural integrity with subunit structure are required for the optimal function of H^+/K^+ ATPase (Ray et al., 1983). The thiol groups present on the outer surface of gastric microsomal vesicles play a vital role in gastric H^+/K^+-transporting ATPase function (Nandi et al., 1983). 2,1-arylpyrrolo[3,2-c] guinolines,13a (SK&F 96356) acts as an inhibitor of gastric acid secretion in different animal model (Keeling et al., 1991). The β-subunit H^+/K^+ ATPase is required for the localization and catalytic α-subunit function (Morley et al., 1992). A detailed analysis of functional properties of and structure of H^+/K^+ ATPase has been reviewed. (Wingo and Armitage, 1993).

6. Other ATPases

In this section an attempt has been made to summarize the available information on membrane proteins with "putative ATP hydrolytic properties". However, since there was

no systematic classification of these proteins, the following presentation is made to characterize these enzymes:

A. DNA-dependent ATPase/RNA-dependent ATPase/P-glycoprotein

Phi 29 RNA (174 nucleotides) present in the prohead of phage plays a vital role in assembly and also as DNA translocating ATPase during the packaging process (Anderson and Bodley, 1990). The bacterial Rec BCD enzyme subunit REC B has been shown to possess DNA-dependent ATPase activity (Hickson et al., 1985). The Rec/b subunit interacts in a specific manner with ATP and functions as a DNA helicase while displacing oligonucleosides annealed to viral DNA in a ATP dependent/orientation specific manner (Boehmer and Emmerson, 1992). A replication factor C has been purified from *S. cerevisiae* (ScRFC) and shown to be associated ATPase activity; this has structure-specific (primer-template) DNA binding activity. In comparison with phage T_4 and SV 40 DNA replication, the yeast replication factor C (RF-C), proliferation cell nuclear antigen (PCNA), replication protein A (RP-A) and DNA polymerase (Delta) function as a part of the DNA replication system complex (Fien and Stillman, 1992).

Rec G, a junction specific DNA helicase, plays a major role in recombination and DNA repair. The mechanism of REGG-mediated DNA repair is through its interaction with Rec A. This mechanism is universal and the gene conversion occurs without any crossing over. In this process, the Rec G counters Rec A mediated DNA strand exchange (Whitby et al., 1993). The eIF-4_A, eukaryotic translation initiation factor, possesses RNA-dependent ATPase and RNA helicase activities. It is essential for binding of eukaryotic mRNA to

ribosomes. Also, eIF-, a subunit of transcription of the initiation factor is also required for the mRNA-ribosome binding. eIF-4_A binds to the mRNA cap structure and plays an essential role in the translation of the uncapped transcripts. The eIF-4_A is as a factor needed for the recycling of the ribonucleoprotein complex during translation (Pause et al., 1994). Vaccinia virus early transcription factor (VETF) binds to the early gene promoters also possesses and DNA-dependent ATPase activity. The latter property has been demonstrated to be a prime requirement for the transcriptional activation. A 25T and V278M were defective in early gene promotor binding but retain ATPase activity with high DNA concentration for ATPase activation (Li et al., 1994).

The putative RNA helicase (DEAD/H family) has been shown to possess RNA dependent ATPase activity, contributing towards the conformational changes in the RNA secondary structure. mDEAD2, mDEAD3 and mDEAD5 are the newly characterized members of this family. The mDEAD2 and mDEAD3 sequences showed high sequence homology with yeast DED1 and DBP1 proteins whereas mDEAD5 showed sequence homology with yeast translation initiation factors (TIF1/TIF2 and mammalian eIF-4A. Thus it has been shown that mDEAD/TIF/DBP1 protein sequences are well conserved and are implicated in the RNA metabolism and regulation of gene expression (Gee and Conboy, 1994). It should be pointed out that RNA processing or mRNA splicing takes place in a ribonucleoprotein complex referred to as spliceosome. RNA-dependent ATPase (DEAD/DEAH proteins) has been shown to play a major role in the spliceosome assembly in RNA splicing. Interaction with U2-U6 helix I structure is a major factor in determining

the role of prp16 mRNA splicing (Madhani and Gutherie, 1994).

p68, a human RNA helicase (nuclear RNA-dependent ATPase), belongs to the DEAD box putative helicase proteins. Its functional roles are translation initiation, RNA splicing and ribosome assembly in eukaryotes. The nucleotide sequence analysis indicate that a protein kinase C phosphorylation site and calmodulin binding (IQ domain) which are similar to the neuromodulin (GAP43) a neural specific protein and neurogranin (RC3). p68 ATPase activity is inhibited by phosphorylation/Ca^{2+}-dependent binding of calmodulin. Thus p68 has been implicated in RNA unwinding activity which in turn is regulated by dual Ca^{2+}-mediated signal transduction (Buelt et al., 1994). A putative ATPase (subunit p45) of 26 S proteosome from human hepatoblastoma has been cloned and sequenced. The sequence analysis indicates that p45 is similar to a yeast transcriptional factor sug1p, and Trip1, a functional equivalent of sug1p. Based on the extensive sequence homology, it was suggested that these two factors may be coded by a single gene functioning in transcription and protein degradation (Akiyama et al., 1995).

It has been shown that Protease La has an essential role in protein degradation in *E. coli*. It possesses two high affinity and two low affinity ATP binding sites. Vanadate is a potent inhibitor which blocked the ATP binding to protease La (Menon and Goldberg, 1987). GroEL/hsp60 are ubiquitous in their presence in mitochondria of prokaryotes and eukaryotes. Their major functional properties are protein folding and targetting to the membranes and also regulation by protein-protein interaction as well as by protein renaturation (De Crouy-Chanel et al., 1995). Heat shock protein 70, plays a vital role as a molecular

chaperone in synthesis, folding and also in translocation of nascent peptides. Furthermore, the regulatory motif present in EEVD, C-terminal region determines the ATPase activity, and intermolecular action with substrates, as well as intermolecular interaction between HSP70 and HDJ-1 (Freeman et al., 1995). RAD3 gene product has a single stranded DNA-dependent ATPase and DNA-RNA helicase and DNA helicase activities. This RAD3 protein is required for DNA excision repair and also it is important for cell viability. A similar protein (rhp^{3+} gene product) is also required for DNA repair and cell viability (Reynolds et al., 1992). The yeast SNF/SWI multiprotein complex assists in several gene specific activators as hypothesized by altering the chromatin structure with release of the repression. SNF2a member of SNF/SWI protein family has been shown to have DNA-dependent ATPase activity (Callsen and Laurent, 1994).

Reverse gyrase, which is an ATP-dependent topoisomerase, has been purified and characterized from the *Methanopyrus khnanleri* (Hyper thermophile). This protein has been shown to play a vital role in forming the positive supercoil of DNA. The purified reverse gyrase is made up of two subunits. Subunit A (150 kD) possesses ATPase activity, whereas subunit B, a 50 kD protein, forming covalent complex with DNA (Kozyavkin et al., 1994). Gene-specific transcriptional activation is mediated by the concerted action of multiprotein complex SNF/SW1 with DNA-dependent ATPase activity. Proteins homologous to the SNF/SW1 have been identified and cloned in yeast, drosophila, mice and human. A nucleosome remodelling factor ISWI, subunit molecule NURF has been characterized. NURF has a sequence homology with SW2/SNF2 in the ATPase domain. The new ISWI

protein is localized in the nucleus of the cell, its expression pattern is evident in the nucleus throughout the development in Drosophila (Tsukiyama et al., 1995). ISWI (140 kD) subunit of NURF as ISWI, a member of SWI 2/SNF 2 ATPase family has been localized in the nucleus and implicated in chromatin remodelling (Tsukiyama et al., 1995).

It has been shown that RNA-dependent ATPases/putative helicase is a requirement for localization of RNA in a region-specific manner during Drosophila oogenesis (Gillespie and Berg, 1995). Based on the amino acid sequence homology analysis, the translation elongation [EF-3] has been proposed as an evolving ribosome-dependent ATPase binding protein which has an intrinsic ATPase activity (Belfield et al., 1995).

An eukaryotic DNA replication factor referred to as proliferating cell nuclear antigen plays a vital role both in DNA replication and a processivity factor of DNA polymerase delta. The replication factor C (RF-C) ATPase activity was attributed to the C-terminal residues Lys 254→Glu 256. However, these amino acid residues were not essential for stimulation of DNA synthesis (Fukuda et al., 1995). RF-C of yeast has been cloned and characterized. RF-C 1 has been shown to be identical to cell division cycle gene coding protein participating in DNA metabolism (CDC44). The complex RF-C 1/CDC44 has been hypothesized to interact with the gene encoding PCNA (Cullman et al., 1995). YDJIp, a homologue of a dnajp (eukaryotic), has been shown to upregulate the heat shock protein 70 homologue of yeast HSP70ssAI. Interaction of YDJIp and HSP 70ssAI is essential for the HSP 70ssAI chaperone activity in eukaryotes (Cyr et al., 1992).

HSP 70, stress proteins including *E. coli* Dna K possesses ATPase activity. HSP 70

and Dna K bind to protein substrate in an ATP-dependent manner. In bovine HSC 70, the Mg^{2+} ATP-unfolded protein substrate-HSC 70 complex formation requires the E175 glutamate residue. Also, the glutamate 171 residue in Dna K is essential for substrate binding with ATPase activity. This step is shown to be required for the chaperone property of Dna K (Buchberger et al., 1994). The N-terminal (14 amino acid) region of *E. coli* Dna B possesses the DNA-dependent ATPase activity and DNA helicase activity (Biswas et al., 1994). G/F motif (Gly/Phe) of Dnba J delta 77-107 modulates Dna K's ability to bind to substrate. However, this G/F motif has no impact on DnaK's ATPase activity (Wall et al., 1995). The *E. coli* heat shock protein composed of Dna K (Hsp 70), DnaJ and Grp E which function as a chaperone machinery including protein folding. Protein substrate chaperone system interaction are regulated by ATPase activity of DnaK. A nucleotide factor GrpE regulates the ATPase activity of DnaK (Schonfeld et al., 1995).

Peptides of 8 or 9 amino acid residues cause 40-fold increase of ATPase activity of Dna K in the GrpE's presence (Jorden and McMacken, 1995). Deletion mutant DnaJ delta 144-200 did not affect the DnaK's ATPase activity nor its binding property with Dna K. However, Dna J delta 144-200 inhibited lambda phage growth by reducing its affinity to bind P1RepA and lambda P DNA (Banecki et al 1996). Dmp R, a transcription activator expressed constitutively. Transcriptional activation is regulated by the effectors in conjunction with its domain structure. Ng et al. (1996) showed that A domain represses the C domain with ATPase activity by direct interaction. Dna B monomers are connected by 45 amino acid linker. A single amino acid substitution in the linker domain showed altered

ATPase activity and lower or no ability to stimulate primase activity. Thus, it was concluded that the linker region possesses the property to influence primase-Dna B interaction. But it is not clear how this property is conferred (Strodal and Maurer, 1996).

B. ABC transporters/traffic ATPases

gp16 (gene product 16), a DNA packaging protein of phage Phi 29 possesses ATPase activity. This property in turn depends on both A-type and B-type ATP binding consensus sequences (ABC transporter). Based on the sequence similarity among phage DNA packaging proteins, phage lambda-gpA, T_7 gp 19 and T_4 g17 are proposed to function as ATPases (Guo et al., 1987). Phage Mu, B-protein component possesses the ATPase activity under *in vitro* conditions. However the critical function of phage Mu, B-protein mediated DNA transfer is not affected by the ATPase activity (Teplow et al., 1988). Imidopeptidase, (E.C. 3.4.13.9) or prolidge, characterized from the human erythrocytes, ha s been identified to be similar to F_1 ATPase (alpha, beta, subunits) at amino acid level (Endo et al., 1996). The binding of template DNA/T_4 gene 45 product cofactor are essential and correlated for the ATPase activity of phage T_4 DNA replication system (Jarvis et al., 1989). Phage T4 capsid protein mature form gp23, a DNA-dependent ATPase, is referred to as a capsizyme. Mutations by amino acid replacement in amber sites reveal partially filled heads. Thus it was hypothesized that gp23 and its expression products play a role in DNA packaging mechanism (Xue and Black, 1990).

Protein ATPase, the gene product of Sec A, SecB and SecY, has been demonstrated as a protein translocater system. The sec A possesses the ATPase activity and acts as a

catalytic subunit of this protein-translocator system (Fandall and Tai, 1990). The ATP binding domain was identified to be present at the amino-terminal segment and it requires the C-terminal region as a structural component. It was also demonstrated that the amino terminal is required for the protein translocation (Matsuyama et al., 1990). On the other hand, periplasmic phosphate permease (Pst system) is a member of the traffic ATPase (ABC cassette) family of transport system. This system is a phosphate-specific transport system. R220 in the pst A protein and R237 and E241 in the Pst C protein were required for inositol phosphate transport (Cox et al., 1988). Double mutation by amino acid substitution in pst A resulted in a permanently closed system. On the contrary, double mutation in the Pst C protein led to the formation of a permanently open transport system (Webb et al., 1992). The Mg^{2+}-transport system in *S. typhimurium* is regulated by three different transport systems. The constitutive Mg^{2+} influx is controlled by corA and a very high $[Mg^{2+}]_o$. It can also mediate Mgt efflux. Mgt A and Mg^{2+} B are expressed only at low extracellular Mg^{2+} concentrations. Nucleoside sequence analysis of Mgt A and Mgt B show that they are similar to P-type ATPase (Maguire et al., 1992).

Bacterial HlyB, a haemolysin exporter is a member of the ATP-binding cassette (ABC) or traffic ATPase superfamily and is structurally-functionally similar to P-glycoprotein. Fusion-protein, expression product of cytoplasmic domain (C-terminal peptide-GST) retained the transferase and ATPase activity. This activity was inhibited by P-type ATPase but not by F- and V-type ATPases, nonhydrolyzable ATP analogues or translocation substrate (Koronakis et al., 1993). Traffic ATPases (ABC) are transporter

proteins, shown to play a vital role in the eye pigment precursor uptake in *Drosophila melanogaster*. It has been demonstrated that the mutant white gene (allele wco2; wBwx) leads to the reduced red pigmentation without interfering with brown pigmentation. This reduced pigmentation resulted in white eye pigmentation color. This has been attributed to the failure of pigment to transport guanine or tryptophane (Ewart et al., 1994).

P-glycoprotein (170 KDa) belongs to the (ATP binding cassette) ABC transporter family and participates in the transport of broad range of amphiphilic and hydrophobic drugs in the tumours. It is a structure with 12 transmembrane helices with two cytoplasmic nucleotide binding domains. Overexpression of carboxyl terminal nucleotide binding domains without transmembrane domain results in low ATPase activity. In fact the transmembrane domain is required for the normal ATPase activity as well as for the drug-induced stimulation (Sharma and Rose, 1995). Hly B, a toxin exporter protein present in *E. coli* possesses an integral membrane domain fused with cytoplasmic terminal of ATP binding cassette (ABC) family participate in haemolysin (110 KDa) export. Amino acid substitutions in the ATP-binding domain/fold and glycine rich region of ABC transporters impairs both exporter and ATPase activity without affecting ATP binding (Koronakis et al., 1995). Mikawa et al. (1995) demonstrated that RecA protein is able to bind to ATP, upon deletion of 33 amino acid residues, but it is unable to participate in self assembly, DNA binding and ATP hydrolysis. However, the deletion mutant protein assembles at higher protein concentration and longer incubation time in the presence of $MgCl_2$ (Mikawa et al., 1995).

C. K^+ ATPase

K^+ ATPase of *Streptococcus faecalis* consists of single peptide with Mr 78 kD and is an ATP-driven pump for the accumulation of potassium by bacterial cell. By immunological screening of the *S. faecalis* genomic library, two genomic clones were obtained. It codes for 583 amino acids with Mr 63 kD. The primary structure of this enzyme exhibits homology with Kdp B subunit of K^+ ATPase of *E. coli* and to a lesser extent with eukaryotic ion motive ATPases (Solioz et al., 1987). Potassium-translocating Kdp ATPase of *E. coli* shares common functional properties with eukaryotic P-type ATPases (Brandt and Neve, 1992). It has been shown that Asp 307 in kdp B is the phosphorylation cation site of the kdp ATPases. Alteration of the amino acid Asp 300→Asn or →Glu showed 30-fold increase of the ATPase activity compared to wild type enzyme (Puppe et al., 1992).

ATP-dependent copper transporting system in isolated plasma membrane fractions of rat liver is distinct from the canalicular glutathione-conjugate transporter (cMOAT) (Dijkstra et al., 1995). Primary structure of Cop A coding for 727 amino acid and Cop B coding for 745 amino acids [P-type ATPases] involved in copper homeostasis indicates that both enzymes have heavy metal ion binding motif in their polar N-terminal region. It has been demonstrated that Cop A and Cop B are heavy metal ion ATPases which regulate the cytoplasmic copper activity with Cop A serving as Cu^{2+}-uptake while Cop B serves in extrusion from the cell (Odermatt et al., 1993).

An accessory protein has been shown to increase the processivity and increase in DNA-holoenzyme complex formation by enhancing the ATP hydrolysis (Jarvis et al., 1991).

Hydrolysis of peptide substrates and the ubiquitinated proteins are carried out by proteosome-PA 700 CPX. It has been shown that PA 700 binds to the proteosome through an ATP-dependent mechanism. PA 700 possesses ATPase activity, and also 4/16 subunits belong to the family of ATP-binding sequences (De Martino and Slanghter, 1993). The proteolytic cleavage product of Rho A (devoid of 15% C-terminal) retains the ATPase activity identical to holo-hexamer Rho A protein complex (Benedict and Kowalczykowski, 1988). Presence of the RNA oligo cofactors at the 5' end of the Rho factor is a prime requirement for its maximum ATPase activity. This property is independent of monovalent or divalent cation concentration (Wang and Van Hippel, 1993). The transcription termination factor rho II is a hexamer, with three strong and three week RNA binding sites for oligonucleotide cofactors. Oligo rc's are required to form Rho-RNA complex and the oligo ru's essential for RNA release from individual cofactor site coupled with ATP hydrolysis (Wang and Van Hippel, 1993).

An enteric bacterial protein, NTRC possesses the enhancer binding property and thereby activates the transcription under limiting conditions. By site-directed mutagenesis, it has been shown the D54→E with S160→F substitution resulted in constitutive ATPase activity and transcriptional activation. Phosphorylation at aspartate 54 is a requirement for NTRC-mediated hydrolysis and also the transcription (Klose et al., 1993). The factor Rho is required for the RNA polymerase-mediated transcription termination in *E. coli.* Rho binds to the purine rich RNA target sites followed by the RNA-dependent ATP hydrolysis and RNA-DNA helicase activity is required for its optimal function (Platt, 1994). The

Mg^{2+}-transport system in *S. typhimurium* is regulated by three different transport systems. The constitutive Mg^{2+}-influx is controversial by Cor A whereas at very higher extracellular Mg^{2+} $[Mg^{2+}]_o$ concentration it is feasible. It can also mediate Mg^{2+}-efflux. Mgt A and Mgt B are expressed only at a low extracellular Mg^{2+} concentrations. Nucleotside sequence analysis of Mgt A and Mgt B show that they are similar to P-type ATPases. A gene coding for heavy metal transporting (copper) ion P-ATPases are linked to Wilson's diseases and Menkes disease (MNK). The candidate gene WD (ATP_7B) has significant homology with X-linked copper transport disorder, i.e. Menkes disorder (Petrukhin et al., 1994).

II. STATEMENT OF THE PROBLEM AND OBJECTIVES

Ca^{2+}/Mg^{2+} ecto-ATPase is present in the heart plasma membrane and requires millimolar concentrations of Ca^{2+} or Mg^{2+} for maximal hydrolysis of ATP. On the basis of the following criteria, Ca^{2+}/Mg^{2+} ecto-ATPase has been classified as an E-type ATPase [E.C.3.6.1.15] (Webb, 1992): i) Ca^{2+}/Mg^{2+} ecto-ATPase has a catalytic site located on the extracellular surface; ii) Ca^{2+}/Mg^{2+} ecto-ATPase is insensitive to inhibitors of **P-type** cation transport ATPases [i.e. Na^+/K^+ATPase, Ca^{2+}-pump ATPase of the sarcolemma and sarcoplasmic reticulum such as ouabain (at 1 mM) and vanadate (50 μM), **F-type** ATPases [i.e. F_1F_0 mitochondria proton pump] such as NaN_3 (5 mM) and oligomycin (6 μM) and **V-type** ATPases [i.e. vacuolar proton pumps] such as N-ethylmaleimide (NEM, 0.5 mM) and fluoride (10 mM); iii) Ca^{2+}/Mg^{2+} ecto-ATPase is inactivated by detergents; iv) Ca^{2+}/Mg^{2+} ecto-ATPase does not form phosphoprotein intermediates during substrate hydrolysis; v) Ca^{2+}/Mg^{2+} ecto-ATPase is dependant upon millimolar (mM) concentrations of substrates as well as Ca^{2+} or Mg^{2+} as a cofactor; vi) Ca^{2+}/Mg^{2+} ecto-ATPase hydrolyzes nucleoside triphosphosphates but not nucleoside diphosphates or nucleoside monophosphates; vii) Ca^{2+}/Mg^{2+} ecto-ATPase has an alkaline pH optimum (pH 7.5 to 8.5); viii) Ca^{2+}/Mg^{2+} ecto-ATPase exhibits a high turnover [formation/breakdown] rate with a calculated turnover number of 500,000 min^{-1} (Treuheit et al., 1992); and, ix) Ca^{2+}/Mg^{2+} ecto-ATPase is present in low abundance in most tissues (Dhalla and Zhao, 1988; 1989; Plesner, 1995; Plesner et al., 1997). A variety of functions have been proposed for Ca^{2+}/Mg^{2+} ecto-ATPase (Dhalla and Zhao, 1988; Plesner, 1995) including termination of purinergic signalling in smooth

muscle cells (Su, 1983), neurotransmission (Burnstock, 1996), non synaptic information transfer (Trams and Lauter, 1974; Edwards et al., 1992), secretion (Beaudoin et al., 1986; Strobel and Rosenberg, 1992), vesicle trafficking (Kittel and Bacsy, 1994), Ca^{2+}-influx and Mg^{2+}-efflux from cardiac myocytes (Dhalla and Zhao, 1989), regulation of ecto-kinase substrate concentration (Desouza and Reed, 1991), cell adhesion (Lin and Guidotti, 1989; Cunningham et al., 1993; Cheung et al., 1993; Kannan et al., 1997; Kirley, 1997; Dzhanzhugazyan and Bock, 1993; 1997a, b; Tingstrom et al., 1990; Knowles et al., 1997), bicarbonate transport (Martin and Senior, 1980), tumourigenesis, (Knowles, 1995), mesentric artery functions (Jule et al., 1993), mechanochemical functions involving cell motility and adhesion in HeLa cells and also in Erlich ascites tumour cells (Epstein and Holt, 1963; Stewart et al.,1969).

Lin and Guidotti (1989) were first to show that expression of cDNA for the Ca^{2+}/Mg^{2+} ecto-ATPase from rat liver produced a protein with cell adhesion properties. Dhanzhugazyan and Bock (1993; 1997b) also reported that neural cell adhesion molecule possesses an E-type ATPase or Ca^{2+}/Mg^{2+} ecto-ATPase activity. Additional support for this viewpoint was obtained by sequence analysis of a cDNA representing the chicken gizzard smooth muscle Ca^{2+}/Mg^{2+} ecto-ATPase which indicated a maximum sequence homology with mouse CD39 (Kansas et al., 1991). Interestingly, CD39 has been identified as a glycoprotein that functions as a surface marker in the Epstein Barr virus transformed B lymphoblastoid cells (Kansas et al., 1991). Although these data appears convincing, it has been postulated that the chicken gizzard smooth muscle Ca^{2+}/Mg^{2+} ecto-ATPase may not be a cell adhesion

molecule (Kirley, 1997; Lewis-Carl and Kirley, 1997). This alternative proposal which was promoted independently by Stout et al. (1994) and Knowles et al. (1997) is based on the concept that the ecto-ATPase and cell adhesion activities exist on separate but tightly associated proteins. Although it was reported that the cardiac Ca^{2+}/Mg^{2+} ecto-ATPase may be a distinct ectonucleotidase with more than one physiological function (Dhalla and Zhao, 1988; Plesner et al., 1997), the molecular structure of Ca^{2+}/Mg^{2+} ecto-ATPase is not known at present. It was therefore thought worthwhile to undertake a systematic analysis for obtaining the information on structural and functional properties of the purified Ca^{2+}/Mg^{2+} ecto-ATPase concerning biophysical, molecular and immunochemical aspects. It is hoped that this investigation will provide further information for elucidating the role of sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase in heart function.

III. METHODS

1. Purification of Ca^{2+}/Mg^{2+} ecto-ATPase

A. Isolation of heart plasma membranes

Plasma membranes were isolated from the rat cardiac tissue by the sucrose gradient density method essentially as described by Pitts (1979). Male Sprague-Dawley rats were decapitated and their hearts were quickly placed in the ice cold homogenizing medium (0.6 M sucrose, 10 mM imidazole-HCl pH 7.0). The cardiac tissue was washed throughly in homogenizing medium, diced with a pair of scissors and homogenized in a polytron PT-20 (Setting 5, 20 sec x 5 with 20 sec intervals) in homogenizing medium. The homogenate was centrifuged at 12,000 X g for 30 min at 4°C. The supernatant was diluted with @ 5 ml/g KCl/MOPS buffer (160 mM KCl, 20 mM MOPS, pH 7.4) and centrifuged at 96,000 x g for 60 min at 4°C. The pellets were suspended in 1 ml/g KCl/MOPS buffer and the suspension was carefully overlaid on sucrose buffer (30% sucrose, 0.3 M KCl, 50 mM sodium pyrophosphate, 0.1 M Tris-HCl, pH 8.3). After centrifugation at 95,000 x g for 90 min 4°C, the transparent-buffer band at the sucrose-suspension interphase was removed, and diluted with 3 volume of KCl/MOPS buffer and centrifuged at 96,000 x g for 30 min at 4°C. The pellet was suspended in 50 mM Tris-HCl, pH 7.4 and utilized as purified plasma membrane for solubilization purposes.

B. Assay of E-type ATPase activity

The E-type ATPase activity in the presence of Ca^{2+} or Mg^{2+} was carried out at 37°C in 1 ml of assay medium containing: 50 mM Tris-HCl (pH 7.4), 4 mM Tris-ATP, 3-5 μg of

purified Ca^{2+}/Mg^{2+} ecto-ATPase or 20-30 μg of total membrane protein and 4 mM $CaCl_2$ or $MgCl_2$. The reaction was initiated by adding ATP and terminated by the addition of 1 ml of Pi reagent (100 ml = 1.0 g $FeSO_4$; 18 ml DDW; 2 ml 10% ammonium molybdate in 10 N H_2SO_4; (prepared fresh)). The Pi estimation was carried out by following the method of the Taussky and Shorr (1953). The protein concentration was determined essentially as described by Lowry et al. (1953).

C. Solubilization of Ca^{2+}/Mg^{2+} ecto-ATPase

The Ca^{2+}/Mg^{2+} ecto-ATPase was purified by following the procedure described by Zhao and Dhalla (1991). Briefly, the sarcolemmal membranes at a final protein concentration of 10 mg/ml were solubilized in 1 ml of a medium containing 5 mg lysophosphatidylcholine, 5 mg CHAPS, 0.6 M NaI, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), at room temperature for 10 min. The non-solubilized material was removed by centrifugation at 100,000 x g at 4°C for 60 min. The supernatant (1 to 1.5 ml) was layered on a sucrose gradient (3 ml each of 15%, 20%, 25%, 30%, 35% and 40% sucrose solution buffered with 50 mM Tris-HCl, pH 7.4) and centrifuged at 160,000 x g overnight (16 hr) at 4°C. Fractions of 1 ml each were collected and those with Ca^{2+}/Mg^{2+} ATPase activity were pooled. The pooled samples were passed through a cibacron blue dye column (0.6 x 15 cm) equilibrated with 50 mM Tris-HCl (pH 7.4). The Ca^{2+}/Mg^{2+} ecto-ATPase was eluted from this column with the equilibrating buffer and the fractions (0.5 ml) with Ca^{2+}/Mg^{2+} ATPase activity were pooled and applied to a sepharose 6B column (1 x 20 cm) equilibrated with 50 mM Tris-HCl (pH 7.4). The Ca^{2+}/Mg^{2+} ecto-ATPase from the sepharose 6B column was

eluted with the equilibrating buffer at the rate of 10 ml/hr and the fractions (0.5 ml) exhibiting Ca^{2+}/Mg^{2+} ATPase activity were pooled and utilized for further studies.

2. Biophysical characterization of rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

A. Electrophoretic analysis

The purified protein was also analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Bands were visualized either by coommassie brilliant blue staining (Wilson, 1983) or by silver staining (Morrissey, 1981) procedure. Gradient gel electrophoresis (10-20%) with 0.5 M Tris-HCl buffer pH 8.3 was carried out according to the procedure described in the Bio-Rad Mini-Protein II Instruction Manual. Gel electroelution was performed by using Hoefer-GE 200, six-Pac gel eluter following the instructions provided by the Hoefer Scientific Instruments (San Francisco, CA). Iso-electric focusing and SDS-PAGE (second dimension) were carried out as per the procedure described by the Bio-Rad Instruction Bulletin 1144.

B: Trypsin digestion, HPLC separation and protein sequencing of Ca^{2+}/Mg^{2+} ecto-ATPase

About 10 μg of the purified cardiac Ca^{2+}/Mg^{2+} ecto-ATPase protein was subjected to 10% SDS-PAGE; the protein was transferred to polyvinylidene difluoride (PVDF) membrane followed by staining with Ponceau S. PVDF slices containing the Ca^{2+}/Mg^{2+} ecto-ATPase protein band (90 kD) were cut out and submitted to in situ digestion with trypsin (Lane et al., 1991). The resulting peptide mixture was separated by narrow-bore high performance liquid chromatography (HPLC) using a Vydac C18 reverse phase column (2.1 X 150 mm) on a

chromatography (HPLC) using a Vydac C18 reverse phase column (2.1 X 150 mm) on a Hewlett-Packard 1090 HPLC with a 1040 diode array detector. Optimum fractions were chosen based on their symmetry, resolution, ultraviolet absorbency and spectra; these were further screened by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/MS) and submitted to automated Edman degradation on an Applied Biosystems 477A protein sequencer (Microchemistry Laboratory, Harvard University, Boston, MA). Strategies for peak selection, reverse-phase separation and protein microsequencing were the same as described by Lane et al. (1991).

C. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF/MS)

The protein was suspended in an appropriate amount of Mill-Q water to obtain a stock concentration of 100 μM. Aliquots of the stock solution were then diluted to give final concentrations of 2 nmol/μl, 20 pmol/μl, 200 pmol/μl and were then added on the sample support with an equal volume of a saturated matrix solution to obtain final protein concentrations of 1 nmol/μl, 10 nmol/μl and 100 pmol/μl, respectively. For each sample, three matrices were used: (a) [2-(4-hydroxy phenylaxol)]-benzoic acid (HABA); (b) 3,5, dimethoxy-4-hydroxy cinnamic acid (SINAPINIC); (c) alpha-cyano-4-hydroxy cinnamic acid (ALPHA), which were dissolved to saturation in 40% aqueous acetonitrile. Analysis was performed on a linear Tofspec MALDI mass spectrometer (VG Analytical, Manchester, UK) at the Department of Chemistry, University of Waterloo (Waterloo, Canada) (Kannan et al., in press).

D. Molecular cloning and characterization of rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

i. Oligonucleotide probes

The purified Ca^{2+}/Mg^{2+} ecto-ATPase was subjected to the in situ tryptic amino acid sequencing and internal amino acid sequence [TYLDVEPITGFTLQFAK] was obtained (Kannan et al., 1997). This sequence was subsequently used to design (via MacVector™) the degenerate oligonucleotides PT-91-A (5'CCAGTTATGGGTTCCACATCTAAG3') and PT-91-B (5'TGGCTAAATGAGACTGGGAC3') [synthesized by University Core DNA service at the University of Calgary (Calgary, Canada)].

ii. Isolation of rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase cDNA

Rat heart 5'-stretch cDNA (male Sprague-Dawley) library in λgt10 (Clontech Inc, Palo Alto, CA, USA) was screened with the oligonucleotide probe PT-91-A essentially as recommended by the manufacturer. Subcloning was carried out by following the procedures described by Sambrook et al. (1988). A single clone designated pND2.1 was obtained and used in the remaining studies.

iii. PCR amplification and sequencing of pND2.1

We were unable to sequence the pND2.1 insert due to its high GC (guanine and cytosine) content. Therefore the cDNA in pND2.1 was subcloned into Bluescript®SK(+) (Stratagene®) and PCR amplified using PT-91-A and oligo-dT as primers. The PCR product was cloned into pCR-script™ SK(+) cloning vector (Stratagene) and sequenced at the Core Facility for Protein/DNA Chemistry at Queens University (Kingston, Canada) using ABI

PRISM™ dye terminator cycle sequencing Kit with AmpliTag^R DNA polymerase, FS. The subsequent reactions were electrophoretically separated and analyzed on an ABI 377 DNA Sequencer system. Nucleotide sequence was analyzed by BLAST™ program.

iv. Blotting procedure

Rat multiple tissue (polyA$^+$) Northern blot™, Interspecies zoo blot™, rat (Sprague-Dawley) GENO-BLOT™, β-actin cDNA and GAPDH cDNA (loading control) were purchased from Clontech Lab Inc. (through BIO/CAN Scientific, Mississauga, Canada). Total RNA was isolated from rabbit, dog and pig cardiac tissue by guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). The final RNA pellet was dissolved in sterile distilled water containing 0.1% diethyl pyrocarbonate (DEPC) and stored at -70°C until further use. Normal human heart total RNA was purchased from Ambion Inc. (Austin, TX). Total RNA was separated on formaldehyde agarose gel and blotted using the procedures described by Sambrook et al. (1988). Blots were probed according to the instructions provided by the manufacturer with pND2.1 radiolabelled with α-^{32}P-dCTP by the random primer method.

v. Protein expression

Rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase cDNA (from pND2.1) was subcloned into open reading frame B or C of pTricHis Xpress™ system and expressed in *E. coli* following the procedures described by Xpress™ system (Invitrogen, Carlsbad, CA, USA). The expression products were analyzed by Western blotting after solubilization of the IPTG (isopropyl β-D-thiogalactopyranoside) induced cells. Immunoblotting was conducted with either

anti-CD36 (Transduction Laboratories, distributed through Bio/Can Scientific, Mississauga, Canada) or anti-rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase (Kannan et al., 1997) as the primary antibodies. Immunoblots were developed as per instructions for the ECL System (Amersham Canada Ltd, Oakville, Canada).

E. Localization of cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase

i. Production of antiserum for Ca^{2+}/Mg^{2+} ecto-ATPase

An antiserum to the purified rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase protein was generated in New Zealand white rabbits by Cedar Lane Laboratories Ltd. (Hornby, Canada). Briefly, a sample of pre-immune serum was collected from two rabbits, prior to the first inoculum being injected. The inoculum was prepared by dissolving 0.2 mg protein in 1 ml phosphate buffered saline (PBS) and mixed with 1 ml complete Freund's adjuvant. Total 2 ml volume of antigen was divided into 4 injection sites (0.5 ml per site). After 40 days, a second injection was given subcutaneously with 0.177 mg protein dissolved in 1 ml PBS and mixed with 1 ml incomplete Freund's adjuvant (0.5 ml per site). After 30 days, a second booster injection was given subcutaneously with 0.26 mg protein dissolved in 1 ml PBS and mixed with 1 ml incomplete Freund's adjuvant (0.5 ml per site). A trial bleeding was performed after 15 days and the presence of the antibody was confirmed by Western immunoblots. A third booster injection was given 15 days later as described above. Final bleeding was performed fifteen days later by total cardiac puncture and the blood was collected. Immunoglobulin (IgG) was purified from the polyclonal antiserum by using Protein A sepharose as described by Harlo and Lane (1983).

ii. Immunoblotting

Purified proteins were subjected to SDS-PAGE and then electroblotted onto Immobilon-P membrane (Millipore, Canada) as described by Kardami and Fandrich (1989). The membranes were shaken for 2 hr in blocking buffer containing 5% fat-free milk in TBS (10 mM Tris, 150 mM NaCl) and then incubated for 1 hr at room temperature with primary antibody (polyclonal rabbit anti-rat cardiac ecto-ATPase) in blocking buffer containing 0.1% Tween-20 and 5% fat free powdered milk. After subsequent washing with TBS containing 0.1% Tween-20 and incubations with secondary antibodies (biotinylated donkey anti-rabbit IgG for 40 min and strepdavidin conjugated horseradish peroxidase solution for 30 min, membranes were rinsed and processed for chemiluminescent detection (ECL kit, Amersham, Arlington Heights, IL).

iii. Immunofluorescence

Sprague-Dawley rat heart tissue sections of 7 μm thickness were obtained using a Leica™ (Wetzlar GMBH, Germany) cryostat and collected on gelatin-coated slides (Kardami and Fandrich, 1989). Sections were placed overnight at 4°C with appropriate primary antibodies diluted in 1% bovine serum albumin (BSA) in PBS and 0.01% (W/V) sodium azide. Secondary antibodies (fluorescein-conjugated, affinity-purified, anti-rabbit IgG (Amersham Corp., Arlington Heights, IL, USA)) were used following the instructions provided by the manufacturer. Sections were counterstained with nuclear fluorescent stain, Hoechst 33342 (bisbenzimide: Behring Diagnostics, La Jolla, CA) as described by Kardami and Fandrich (1989). To view and photograph the selected fields, the UFX-IIA Nikon

system and TX-400 B & W film (Kodak) were used. Immune/pre-immune sera were used at 1:1000>1:20,000 dilution while purified IgG was used at 1-10 μg/ml.

Neonatal cardiomyocytes were obtained from one day old rat pups, and the cultured myocytes were fixed (Kardami and Fandrich, 1991) before staining and were doubly labelled by simultaneous incubation with a monoclonal antibody preparation against Vimentin (clone #V9, Sigma, St. Louis, MO, USA) according to the manufacturer's instruction. Texas-red conjugated anti-mouse IgG was purchased from Amersham (Arlington Heights, IL) and used as described by Kardami and Fandrich (1989). The antiserum at 1:1000 dilution was tested against a range of concentrations for the antigen (1 ng-1 μg) and was found to be capable of detecting 10 ng antigen by immunoblotting. Various dilutions of antiserum (1:1000-1:50,000) were tested for their ability to detect 100 ng antigen. A 1:20,000 dilution was sufficient for detection.

iv. Inhibition of Ca^{2+}/Mg^{2+} ecto-ATPase activity by the anti-Ca^{2+}/Mg^{2+} ecto-ATPase

The ability of anti-cardiac ecto-ATPase to recognize the Ca^{2+}/Mg^{2+} ATPase present in the purified preparation and to inhibit the Ca^{2+}/Mg^{2+} ATPase activity was analyzed by, using IgG from immune or non-immune serum. One μg of purified ecto-ATPase was incubated with or without IgG (0.1-0.5 mg) from immune or non-immune serum in 1 ml of reaction medium (10 mM) Tris-HCl (pH 7.4), 4 mM $CaCl_2$ or 4 mM $MgCl_2$ and 20 μl of 50% protein A sepharose 4B for 16-18 hr at 4°C with constant mixing. The samples were centrifuged for 15 sec (Lin, 1989). The Ca^{2+}/Mg^{2+} ATPase activity was measured in

supernatant and in protein A sepharose 4B pellet suspension in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} with 4 mM Tris-ATP at 37°C (Zhao and Dhalla, 1991).

v. Immunoabsorption

The anti-rat ecto-ATPase serum (1:1000) was incubated with 1 μg of pure ecto-ATPase in 0.1% BSA/PBS for 2 hr at room temperature under gentle agitation in 0.1 ml. The mixture was clarified by centrifugation (10,000 x g, 15 min) and added to tissue sections which were then incubated overnight at 4°C and processed for immunofluorescence. Non-absorbed immune serum was used as a positive control at a similar concentration.

F. Adhesion assay in neonatal cardiomyocytes

i. Cardiomyocyte isolation

Neonatal cardiac myocytes were isolated and characterized from the hearts of one day old rats as described by Pasumarthi et al. (1996). Myocytes were plated in 24 well collagen-coated dishes (100,000 cells/well) in medium F-10 (Gibco BRL) containing 10% fetal calf serum and the cells were left to attach for 4 hr at 37°C. The medium and non-attached cells were removed while the remaining cells were gently rinsed twice with medium F-10 and incubated with 0.5-2.0% fetal calf serum in medium F-10 in the presence of 100 μg/ml of the purified IgG derived from immune or pre-immune rabbit anti-cardiac Ca^{2+}/Mg^{2+} ecto-ATPase serum. These cells were rinsed and processed for PAS staining (Sigma PAS-staining kit) 44 hr later. In another set of experiments, myocytes were plated directly after isolation in the presence of 100 μg/ml of immune or pre-immune IgG and 0.5% fetal calf serum in

medium F-10, the medium and non-attached cells removed 48 hr later and examined as indicated before (Kannan et al., 1997).

ii. Immunocrossreactivity (Ca^{2+}/Mg^{2+} ecto-ATPase and CD36)

Purified Ca^{2+}/Mg^{2+} ecto-ATPase and Jurkat cell lysate (Transduction Laboratories, Lexington, KY, USA) samples were separated by SDS-PAGE, and electroblotted onto immobilon-P membrane (Millipore, Canada) (Kardami and Fandrich, 1989). The membranes were shaken for 2 hr in blocking buffer which contained Tris-saline (10 mM Tris-HCl, 150 mM NaCl) and 5% fat free milk, and then incubated for 1 hr at room temperature with primary antibody (anti-cardiac Ca^{2+}/Mg^{2+} ecto-ATPase serum; 1:1000 dilution) or anti-human CD36 (monoclonal antibodies; 1:1000 dilution) or (pre-immune serum; 1:1000) in Tris-saline buffer containing 0.1% Tween-20 and 5% fat-free powdered milk. After subsequent incubations with secondary antibodies such as biotinylated donkey anti-rabbit IgG, for 40 min and strepdavidin-conjugated horseradish peroxidase solution (Amersham, Oakville, Canada) for 30 min at dilution specified by the manufacturer, membranes were rinsed and processed for chemiluminescent detection (ECL kit, Amersham, Oakville, Canada) (Kannan et al., 1997).

IV. RESULTS

1. Biophysical characterization of rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

A. Structural properties of the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

In one series of experiments, some of the physio-chemical properties and subunit structure of the purified rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase were determined. The isoelectric point (pI) of the purified Ca^{2+}/Mg^{2+} ecto-ATPase protein was found to be 5.7 (Fig. 1). The purified rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase was observed to exhibit a single band at MW 90 kD in the SDS-PAGE (Fig 2, Lane 1). Analysis of the electroeluted enzyme protein band in SDS-PAGE showed two separate bands migrating at MW ~90 kD and 85 kD (Fig. 3, Panel A: Lane 2). Similar results were obtained when the electroeluted protein was subjected to the gradient gel electrophoresis (Fig. 3, Panel B: Lane 2). In order to compare the properties of purified and electroeluted Ca^{2+}/Mg^{2+} ecto-ATPase, the enzyme and the electroeluted proteins were subjected to two dimensional electrophoretic analysis. The results show that the purified enzyme (Fig. 4) was made up of two subunits. Similar results were obtained when the electroeluted protein was analyzed by two dimensional electrophoresis. All our efforts to carry out a complete amino acid analysis of this enzyme by different procedures (Fernandez et al., 1992; Lane et al., 1991) failed.

B. Mass spectroscopic analysis

Mass spectroscopic analysis of purified Ca^{2+}/Mg^{2+} ecto-ATPase was carried out by using three matrix supports: (a) [2-(4-hydroxy phenylaxol)]- benzoic acid (HABA); (b) 3,5, dimethoxy-4-hydroxy cinnamic acid (SINAPINIC); and (c) alpha-cyano-4-hydroxy cinnamic

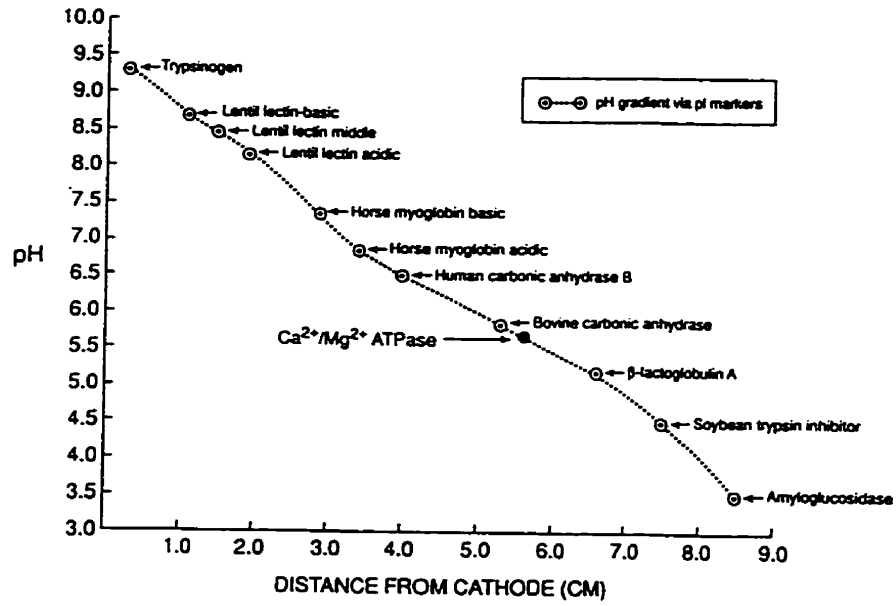

Figure 1. Determination of isoelectric point of the purified rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase using Pharmalyte 3-10 (Pharmacia) on 4% non-equilibrium pH gradient electrophoresis. The comparison values obtained by this method for several other proteins are shown on the curve obtained by blotting pH values and the movement of the protein.

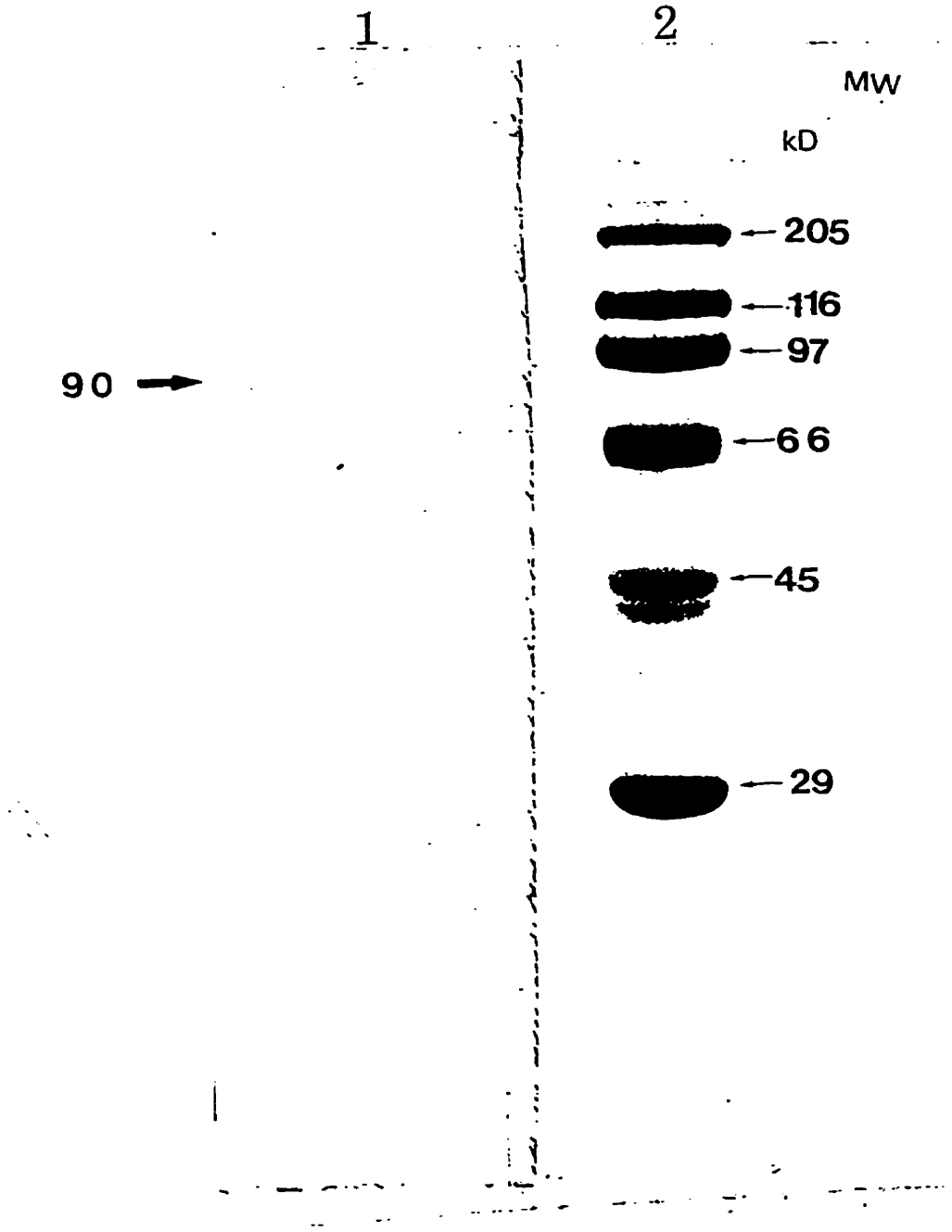

Figure 2. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10%) of the purified rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase (Lane 1). High molecular weight markers (Lane 2).

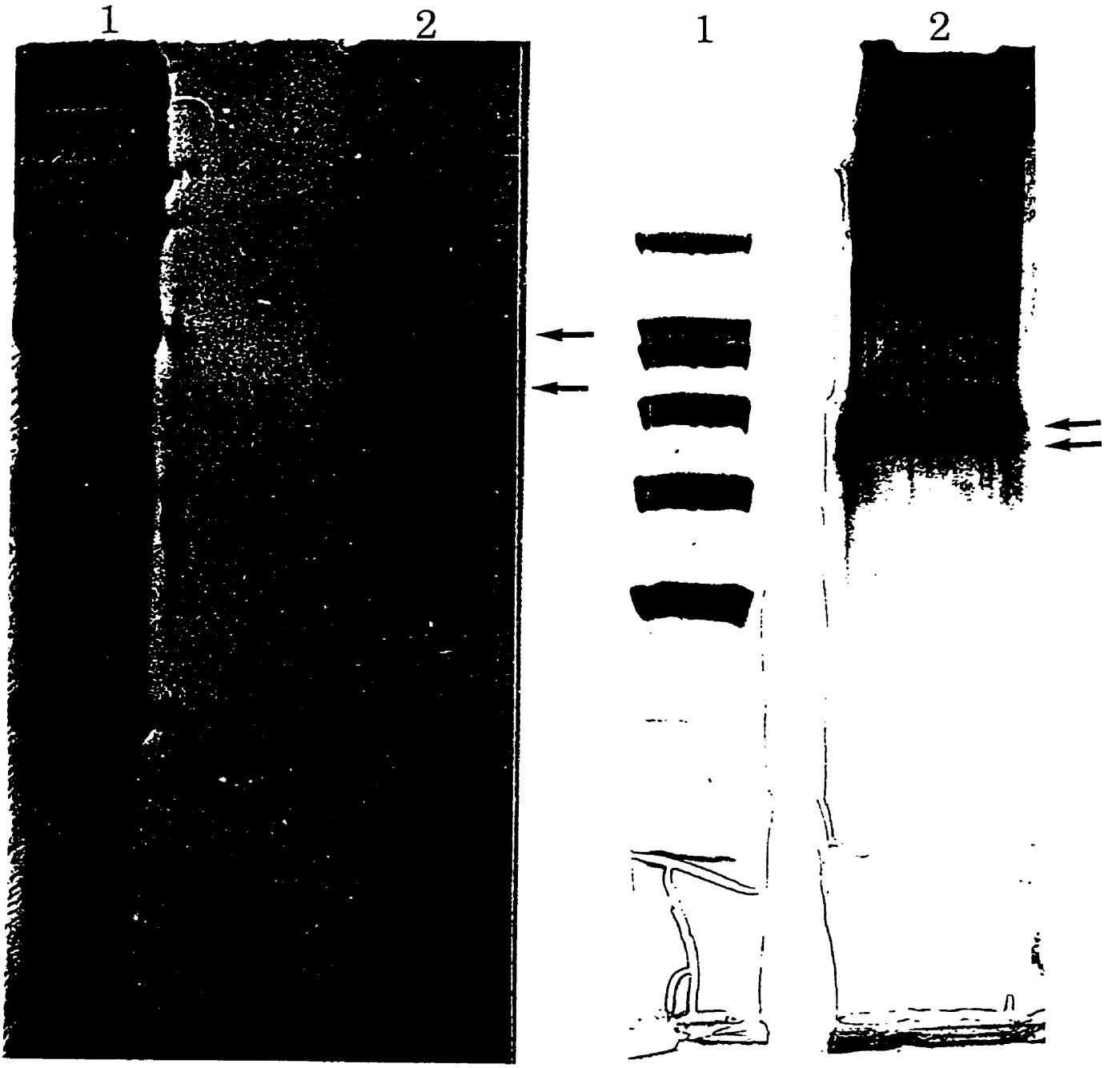

Figure 3. Analysis of the electroeluted Ca^{2+}/Mg^{2+} ecto-ATPase band (90 kD). **Panel A.** Lane 1: molecular weight markers; Lane 2: electroeluted Ca^{2+}/Mg^{2+} ecto-ATPase band (90 kD) separated on 10% SDS-PAGE. **Panel B.** Lane 1: molecular weight markers; Lane 2: electroeluted Ca^{2+}/Mg^{2+} ecto-ATPase band (90 kD) was separated on gradient gel electrophoresis.

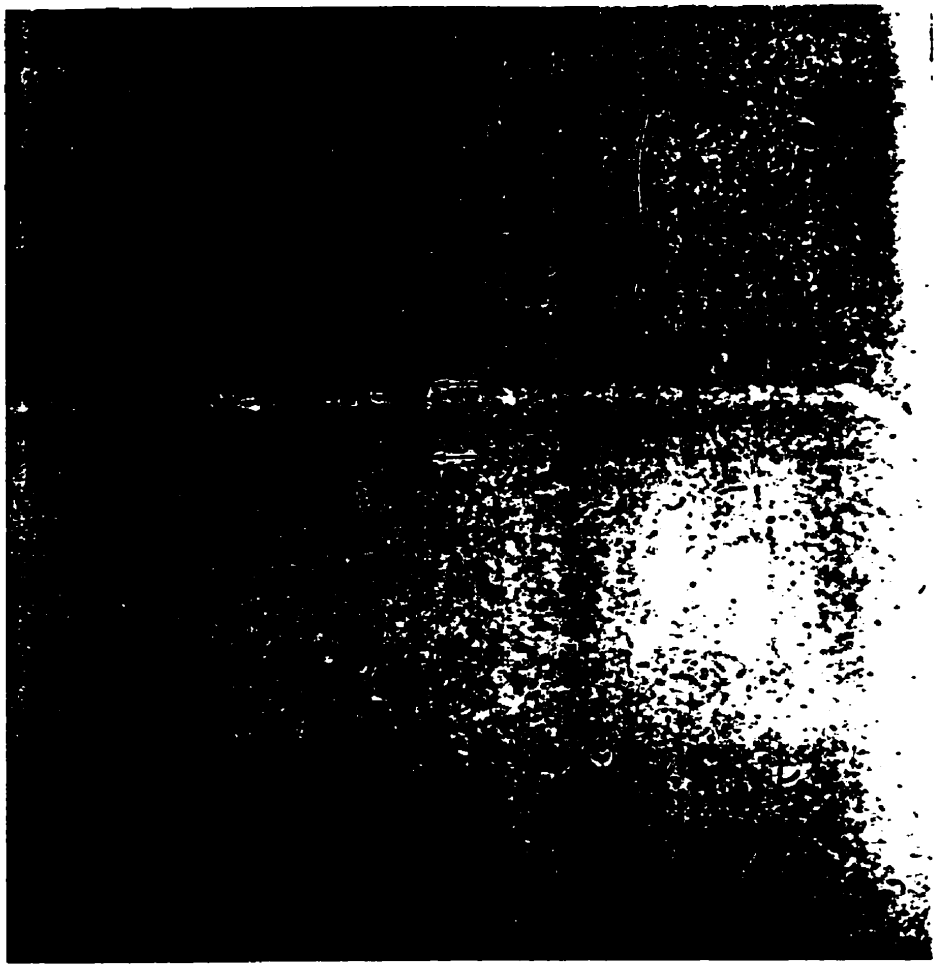

Figure 4. Two dimensional electrophoretic analysis of purified rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase.

acid (ALPHA). In all three matrix combinations analyzed we observed multi-component species of different molecular masses ranging from 1 kD to 5 kD, an indication of microheterogeneity in the enzyme (Fig. 5A, B and C). Pretreatment of the Ca^{2+}/Mg^{2+} ecto-ATPase with DL-dithiothreitol did not modify the pattern of mass spectroscopic analysis.

2. Molecular cloning and characterization of rat cardiac SL Ca^{2+}/Mg^{2+} ecto-ATPase

A. Protein sequence of Ca^{2+}/Mg^{2+} ecto-ATPase

The rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase was purified to homogeneity from cardiac light SL membrane (Zhao and Dhalla, 1991). N-terminal glycosylation of the purified rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase (Zhao and Dhalla, 1991; Kannan et al., 1997) necessitated trypsin digestion and subsequent amino acid analysis to obtain internal amino acid sequence (Fig 6A). The limited sequence information (17 amino acid residues) indicated that there was no sequence similarity with any of the previously reported ecto-ATPases such as CD39 (Kansas et al., 1991), chicken gizzard smooth muscle ecto-ATPase (Kirley, 1997) or the nucleotide triphosphatase of *Toxoplasma gonidi* (Asai et al., 1996). On the other hand, the protein data bank search revealed that the Ca^{2+}/Mg^{2+} ecto-ATPase amino acid sequence was maximally homologous with human platelet CD36, a protein which functions as a receptor for the parasite *Plasmodium falciparum.* Homology (~ 40%) with P-type (SR and SL Ca^{2+}-pump ATPase) ion transport ATPases was also detected (data not shown).

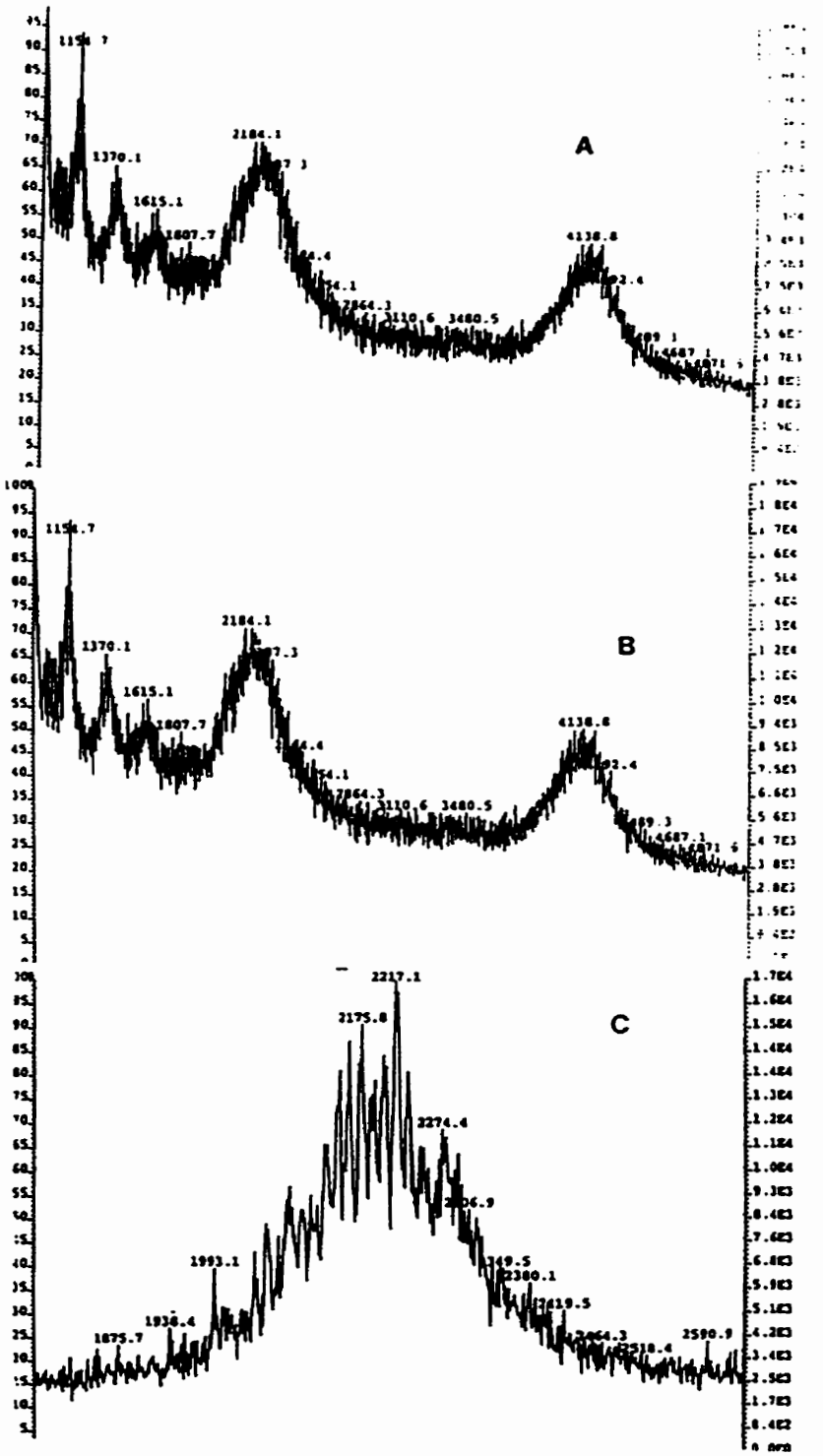

Figure 5. MALDI-mass spectroscopic analysis of rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPases with different matrix combinations. Mass spectrograph of the ecto-ATPase with matrix A: [2-(4-hydroxy phenylaxol)]-benzoic acid (HABA); B: 3,5-dimethoxy-4-hydroxy cinnamic acid (SINAPINIC); C: alpha-cyano-4-hydroxy cinnamic acid (ALPHA).

Panel A:

rSEA	**TYLDVEPITGFTLQFAK**
mCD36	**TYLDVEPITGFTLQFAK**
HPIIb/GPIV	**TYLD i EPITGFTLQFAK**
HACD(c13)	**TYLD i EPITGFTLQFAK**
HACD(c21)	**TYLD i EPITGFTLQFAK**
rFATmRNA	**TYLD i EPITGFTLQF sK**
HACDb	**TYLDuq PITGFTLQFAK**

Panel B:

PT-91-A (5' CCA GTT ATG GGT TCC ACA TCT AAG3')

PT-91-B (5' TGG CTA AAT GAG ACT GGG AC3')

Figure 6. Panel A. Rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase amino acid sequence and analysis: Protein sequence data bank comparison of the rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase amino acid sequence (Internal amino acid sequence: 17 residues) where rSEA = rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase; mCD36 = mouse CD36 antigen cDNA; HPIIB/GPIV = human antigen CD36; HACD (c13) = human antigen CD36 (clone 13); HACD(c21) = human antigen CD36 (clone 21); rFATmRNA = rat fat mRNA gene; HACDb = human antigen CD36(clone b). The amino acid sequence homology was performed by BLAST™ program (for amino acid sequence), National Centre for Biotechnology Information (Bethesda, USA). **Panel B.** The degenerate oligonucleotide probe(s) sequences designed (via MacVector™): PT-91-A (5'CCAGTTATGG GTTCCACATCTAAG3') and PT-91-B (5'TGGCTAAATGAGACTGGGAC3') were synthesized by University Core DNA service at the University of Calgary

B. Isolation of putative Ca^{2+}/Mg^{2+} ecto-ATPase cDNA

The cardiac Ca^{2+}/Mg^{2+} ecto-ATPase amino acid sequence [TYLDVEPITGFTLQFAK] was used to design two different oligonucleotide probes: PT-91-A (5'CCAGTTATGG-GTTCCACATCTAAG3') and PT-91-B (5'TGGCTAAATGAGACTGGGAC3') (Fig 6B). Screening a rat heart cDNA library with PT-91-A yielded seven putative cDNA clones. The cDNA insert present in four clones were similar (~1.0 kB) and a restriction digest produced an identical pattern. Two clones had cDNA insert size of approximately 2.1 kB and these also had a similar restriction enzyme digestion pattern. The remaining clone had an insert of only ~0.5 kB. We chose one of the large cDNA clones (designated as pND2.1 kB) for further studies because of its strong hybridization signal. Since an attempt to obtain clones with a larger cDNA insert (i.e. > 2.1 kB) by rescreening the same cDNA library with pND2.1 kB was unsuccessful, pND2.1 was sequenced. The nucleotide sequence of the pND2.1 insert showed 130% homology to human platelet CD36 (Fig 7A). A high degree of homology (> 70%) with other cell adhesion molecules was also noted (Fig 7B). It should be pointed out that in accordance with the protein sequence, there was no nucleotide sequence homology with chicken gizzard smooth muscle ecto-ATPase (Kirley, 1997), CD39 (Kansas et al., 1991) or *Toxoplasma gonidi* NTPase (Asai et al., 1995).

C. Tissue distribution of Ca^{2+}/Mg^{2+} ecto-ATPase cDNA expression

Since expression of chicken gizzard smooth muscle ecto-ATPase cDNA in other tissues has not been determined, the distribution pattern of Ca^{2+}/Mg^{2+} ecto-ATPase cDNA in mammalian tissues could not be extrapolated. To address this issue, a rat multiple tissue

Panel A:

5' GGAACATAGA AGATTTTGAC TTGTTAATAA GTGAATTGAG
GATCATTTGA ACAAATTGAT TTTGATAGAT ATGGGATGCA
GCCTACAAAA ACAAAGTCAT GCCACAGCCA GATTGAGAAC
TGTGAAGTTG TCAGCCTCTG TTCCAACTGA TAGTGAAGGT
TCGAAGATGG CACCATTGGG CTGCAGGAAA GAGACTGTGT
TGTCCTCAGC GTCCTGGGTT ACATTTTCCT TGGCTAGAAA
ACGAACTCTG TACGTATAAG GACCTCTTTG CTTAACTTGA
ATGTTGCTGC TGTTCATCAT CACTTCCTGT GGATTTTGCA
CATCAAAGAT CCAAAACTGT CTGTAAACTT CTGTGCCCTGT
TTTAACCCAA TTTTTAAAAG CAATTGTACC TTCTTCGAGG-3'

Panel B:

Sequence	Accession numbers	High scores
CD36 antigen, human	(m24795)	1416
CD36 antigen, human	(L06850)	1425
CD36 antigen, human (clone 13)	(m98398)	1416
CD36 antigen, human (clone 21)	(m98399)	1416
CD36 glycoprotein GPIIIb/GPIV human	(S67532)	1407
Rat fat gene (mus musculas)	(L19658)	1049
PAS-4, bovine	(D45364)	1038
CD36 antigen, mouse	(L23108)	993
Lysosmal sialoglycoprotein, human	(D126676)	207

Figure 7. Panel A. Partial nucleotide sequence of the rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase cDNA obtained from clone pND2.1. **Panel B.** Comparison of the partial Ca^{2+}/Mg^{2+} ecto-ATPase cDNA (Clone ND2.1) nucleotide sequence with the gene bank was performed by BLAST™ program at the National Center for Biotechnology Information, NIH (Bethesda, USA). Sequences with high homology as determined by score are presented with the corresponding accession number.

poly A^+ mRNA blot was probed with both the oligomer probe PT-91-A and pND2.1. The results show that the oligomer probe utilized for screening the cDNA library detected multiple transcripts in different tissues. PT-91-A detected a 4.4 kB transcript in heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis. Prominent transcripts of 3.1 kB, ~2.0 kB and ~1.0 kB were also evident in the heart, while a 3.1 kB transcript was detectable in lung, liver and skeletal muscle (Fig 8A). In contrast, pND2.1 detected a single 3.1 kB transcript in cardiac tissue (Fig 8B).

D. Interspecies detection of Ca^{2+}/Mg^{2+} ecto-ATPase sequences

To establish that the putative Ca^{2+}/Mg^{2+} ecto-ATPase cDNA sequence exists in other species, an interspecies genomic DNA Southern blot was probed with pND2.1. This probe detected sequences corresponding to the cDNA present in pND2.1 in humans, rats, bovines and yeast (Fig 9). These results indicate that sequences corresponding to the putative Ca^{2+}/Mg^{2+} ecto-ATPase are present in human, rat, bovine and yeast. The molecular sizes of the detected hybridization signals differed (~3 kB to 7 kB), indicating a diversity in the genomic fragment pattern.

E. Interspecies expression of Ca^{2+}/Mg^{2+} ecto-ATPase mRNA

For the interspecies, expression analysis of mRNA for Ca^{2+}/Mg^{2+} ecto-ATPase was conducted. For this purpose total RNA was prepared from the heart tissue of 4 species and blotted to nylon membrane. Probing this cardiac tissue total RNA blot with pND2.1 detected a ~2.0 kB transcript in canine, rabbit and porcine heart, whereas transcripts of 4.1 kB, ~3.0 kB, and 2.1 kB were observed in human cardiac tissue (Fig 10). These findings are in

Figure 8. Panel A. Tissue distribution analysis: Rat multiple tissue poly A^+ blot (purchased from Clontech Inc. USA) probed with the oligomer PT-91-A as a probe. Each lane contains approximately 2 μg of poly A^+ RNA isolated from the following rat tissues: Lane 1: heart; Lane 2: brain; Lane 3: spleen; Lane 4: lung; Lane 5: liver; Lane 6: skeletal muscle; Lane 7: kidney; and Lane 8: testis. The blot was hybridized for 16 hr at 42°C with ^{32}P end-labelled PT-91-A, in Express Hyb solution. Washes were carried out under stringent conditions (0.1X SSC, 0.1% SDS, 55°C) for 1 hr. X-ray film was exposed at -70°C for 16 hr with two intensifying screens. β-actin (cDNA 2.0 kB) was radiolabelled by the Random Primer method, and utilized as a loading control for the polyA^+ RNA blot. The sizes of the transcripts in the poly A^+ RNA blot were estimated in comparison with the standard RNA markers. The position of the markers was provided by Clontech Inc., as a photographic image of the ethidium bromide stained gel. **Panel B.** Tissue distribution analysis: Rat multiple tissue poly A^+ blot was probed for 16 hr at 42°C with the radiolabelled pND2.1. Washes were carried out under stringent conditions (0.1X SSC, 0.1% SDS, 55°C) for 1 hr. X-ray film was exposed at -70°C for 16 hr with two intensifying screens. The sizes of the transcripts in the poly A^+ blot were estimated in comparison with the standard RNA markers.

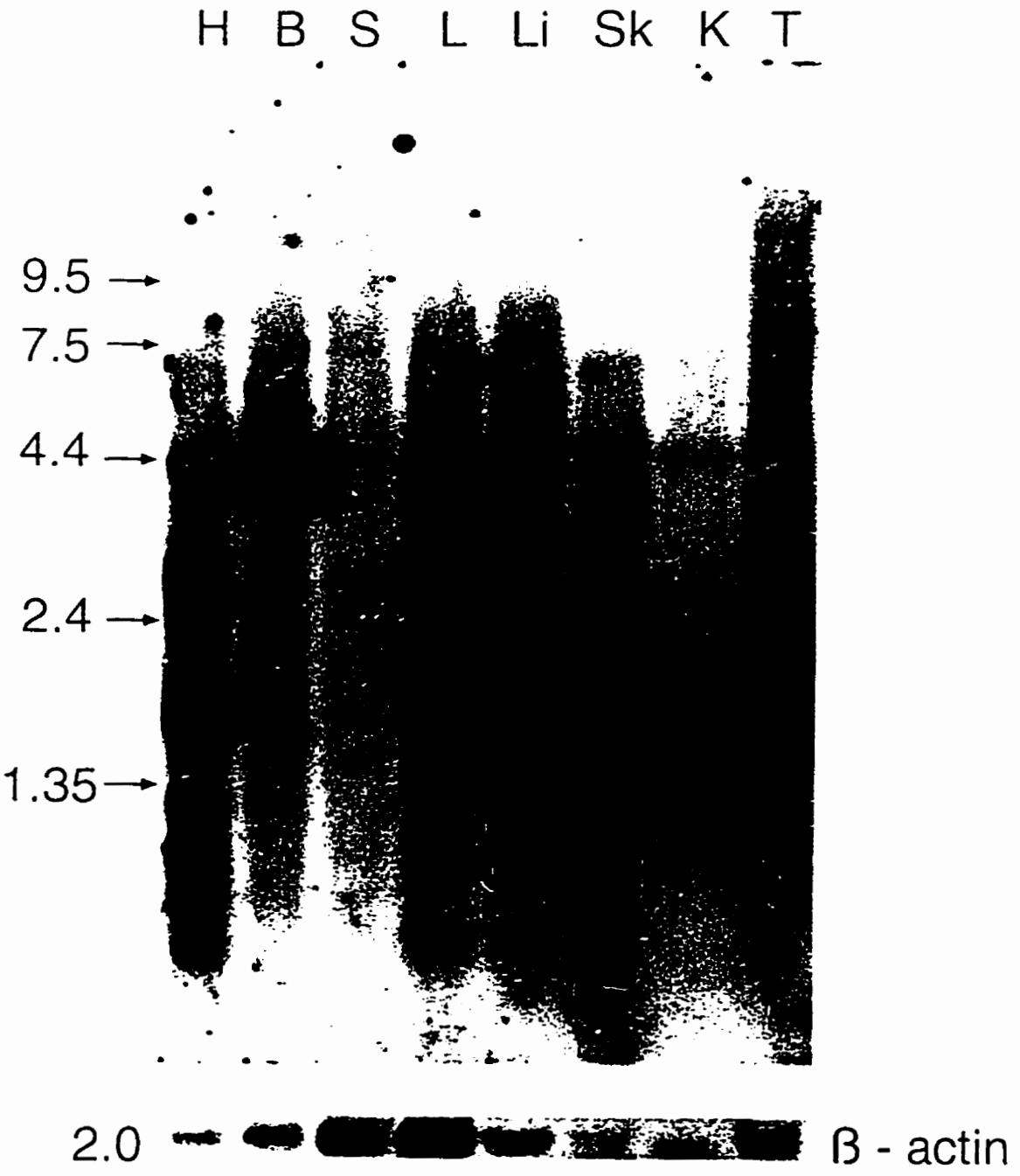

Figure 8 - Panel A

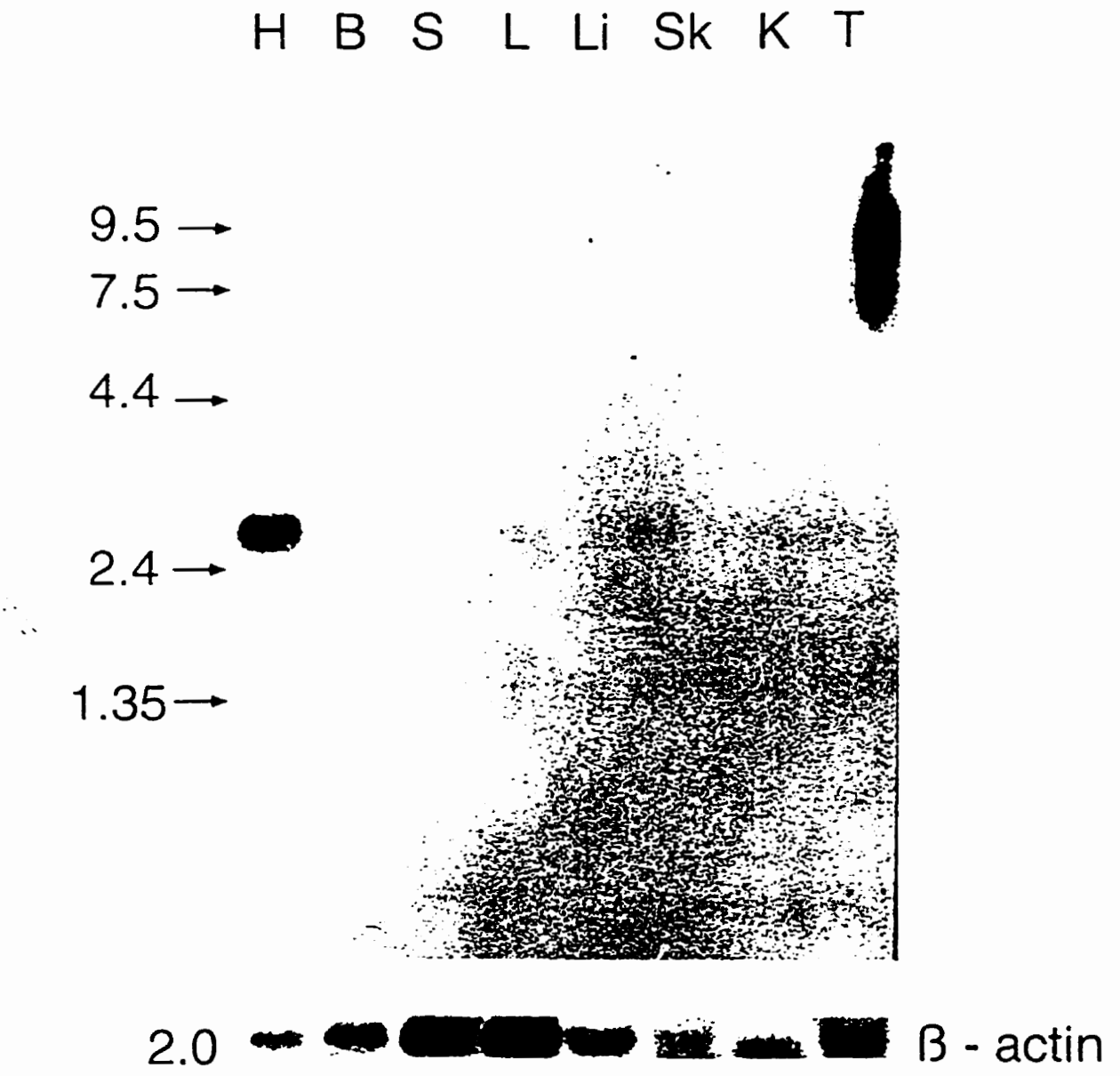

Figure 8 - Panel B

Figure 9. Hybridization of a ZOO-BLOT (Southern blot) with a rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase cDNA pND2.1. **Panel A.** Ethidium bromide-stained agarose gel of a ZOO-BLOT prior to blotting onto a nylon membrane. Each lane contains 4 μg of genomic DNA isolated from the following species: Lane 1: human; Lane 2: monkey; Lane 3: rat; Lane 4: mouse; Lane 5: dog; Lane 6: bovine; Lane 7: rabbit; Lane 8: chicken; Lane 9: yeast. Markers: λ/Hind III DNA size standard. The genomic DNA obtained from different species was digested with EcoRI and resolved on a 0.7% agarose gel electrophoresis in TBE buffer (Tris-Borate-EDTA). **Panel B.** The DNA was transferred from the agarose gel to nylon membrane and hybridized with pND2.1 for 16 hr at 42°C. Washes were carried out under stringent conditions (0.1X SSC, 0.1% SDS, 55°C). X-ray film was exposed at -70°C for 5 days with two intensifying screens.

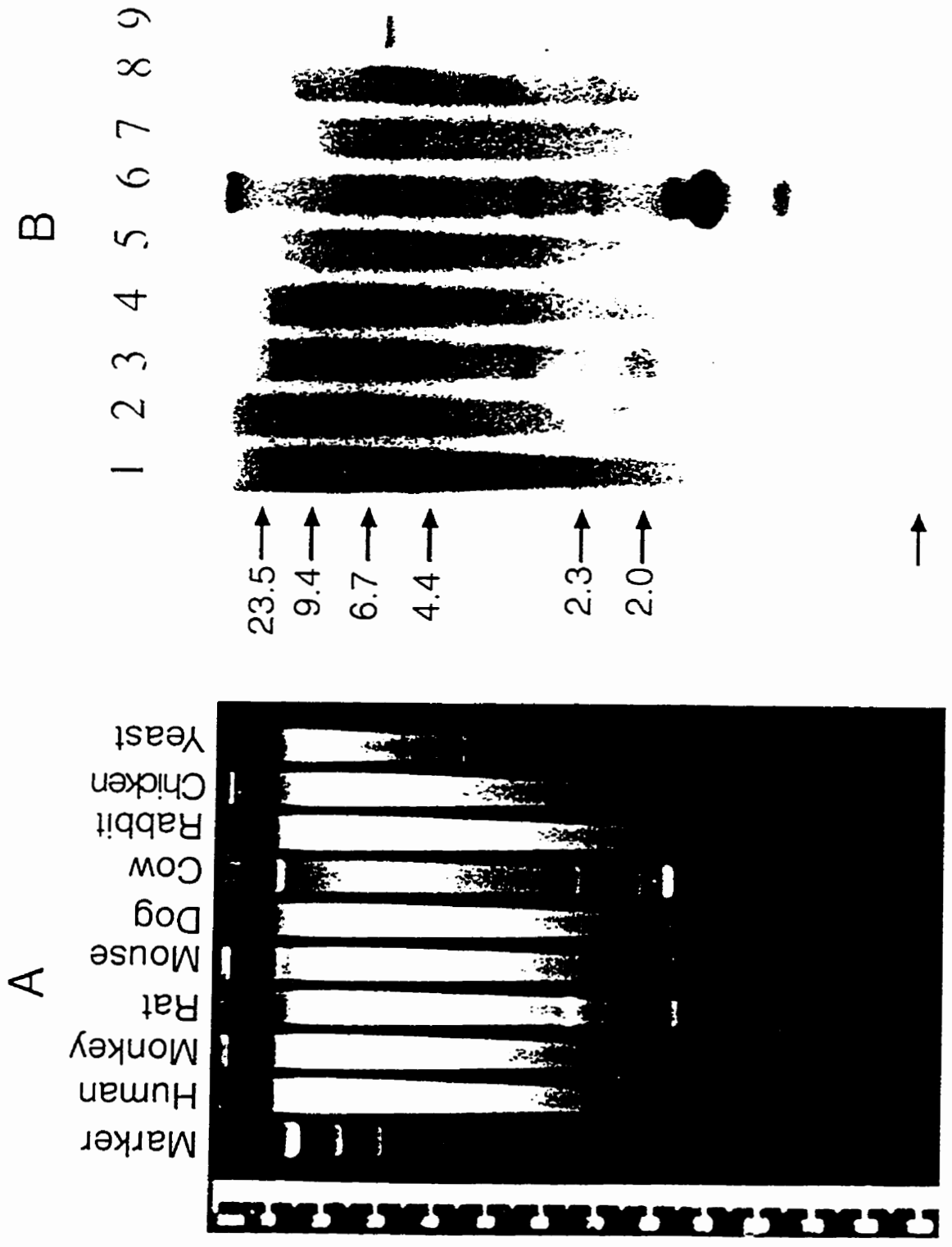
A
Marker
Human
Monkey
Rat
Mouse
Dog
Cow
Rabbit
Chicken
Yeast
B
1 2 3 4 5 6 7 8 9
23.5
9.4
6.7
4.4
2.3
2.0

Figure 9

Figure 10. Hybridization of a ZOO-BLOT (total RNA blot) with rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase cDNA from pND2.1. Each lane contains 20 μg of total RNA isolated from different species resolved by 1.2 % agarose gel containing 1 M formaldehyde. Lane 1: dog; Lane 2: human; Lane 3: rabbit; Lane 4: pig. This blot was hybridized for 16 hr at 42°C with the radiolabelled pND2.1. Radiolabelled GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA was utilized as a loading control, hybridized for 16 hr at 42°C. Washes were carried out under stringent conditions (0.1X SSC, 0.1% SDS, 55°C). X-ray film was exposed at -70°C for 16 hr with two intensifying screens.

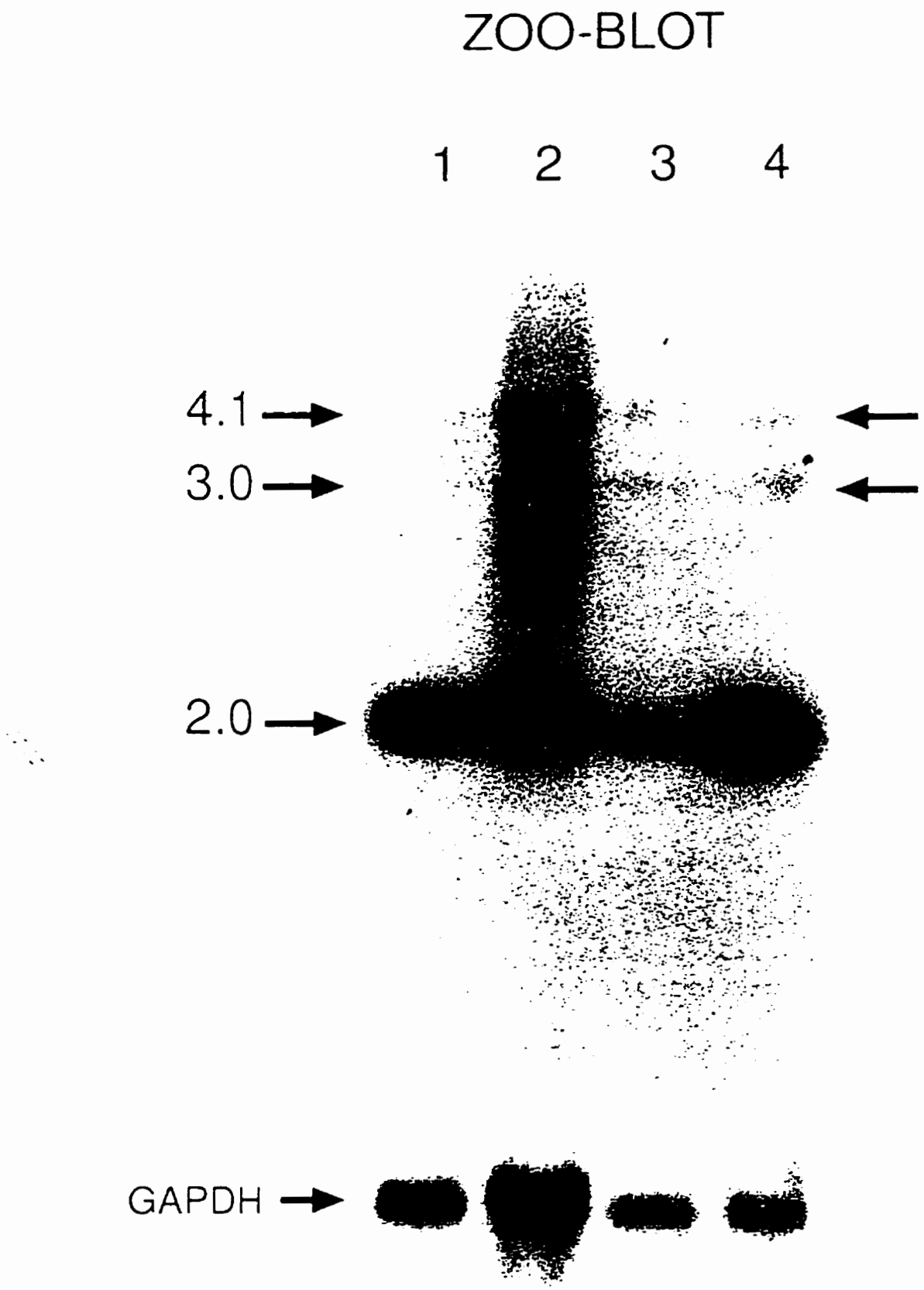

Figure 10

agreement with the multiple tissue blot of RNA.

F. Ca^{2+}/Mg^{2+} ecto-ATPase gene copy number

To determine the gene copy number of the putative Ca^{2+}/Mg^{2+} ecto-ATPase, a rat genomic DNA [digested with different restriction enzymes] blot was probed with pND2.1. This probe detected a signal copy corresponding to a single genomic DNA fragment of varying sizes (genomic DNA digested with Eco RI, ~ 4.4 kB; Hind III, 6.7 kB; BamHI 2 kB; Bgl II ~ 4 kB). These results indicate that a single copy of the sequences corresponding to the putative Ca^{2+}/Mg^{2+} ecto-ATPase are present in the rat genome (Fig 11).

G. Expression and characterization of a putative Ca^{2+}/Mg^{2+} ecto-ATPase cDNA

In an effort to characterize the expression product of Ca^{2+}/Mg^{2+} ecto-ATPase cDNA fragment, it was subcloned from pND2.1 into the expression vector pTricHis. Three distinct open reading frames were present in this plasmid to ensure inframe cloning of the cDNA. Two distinct clones were prepared. The solubilized cell extract obtained after transformation with the orf B clone showed a prominent protein with an Mr 89 kD. This band was absent in untransformed *E. coli.* Western blot analysis subsequently showed that this protein was recognized by the anti-human CD36 monoclonal antibody, but not by an antibody raised to rat Ca^{2+}/Mg^{2+} ecto-ATPase (Fig 12). A parallel analysis of the orf C clone showed that a comparable protein was not expressed upon transformation with this plasmid (data not shown). In agreement with this observation, neither anti-CD36 nor anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase antibodies detected a protein in this extract (data not shown).

Figure 11. Hybridization of a rat genomic Southern blot with rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase cDNA. **Panel A.** Ethidium bromide-stained agarose gel of the rat genomic blot prior to blotting onto a nylon membrane. **Panel B.** Rat genomic blot was probed for 16 hr at 42°C with the radiolabelled pND2.1. Washes were carried out under stringent conditions (0.1X SSC, 0.1% SDS, 55°C). X-ray film was exposed at -70°C for 72 hr with two intensifying screens. Each lane contains 4 μg of rat genomic DNA digested with the indicated restriction enzyme. Lane 1: EcoRI; Lane 2: Hind III; Lane 3: BamH I; Lane 4: PstI; Lane 5: Bgl II; Lane M: λ/Hind III DNA size standard.

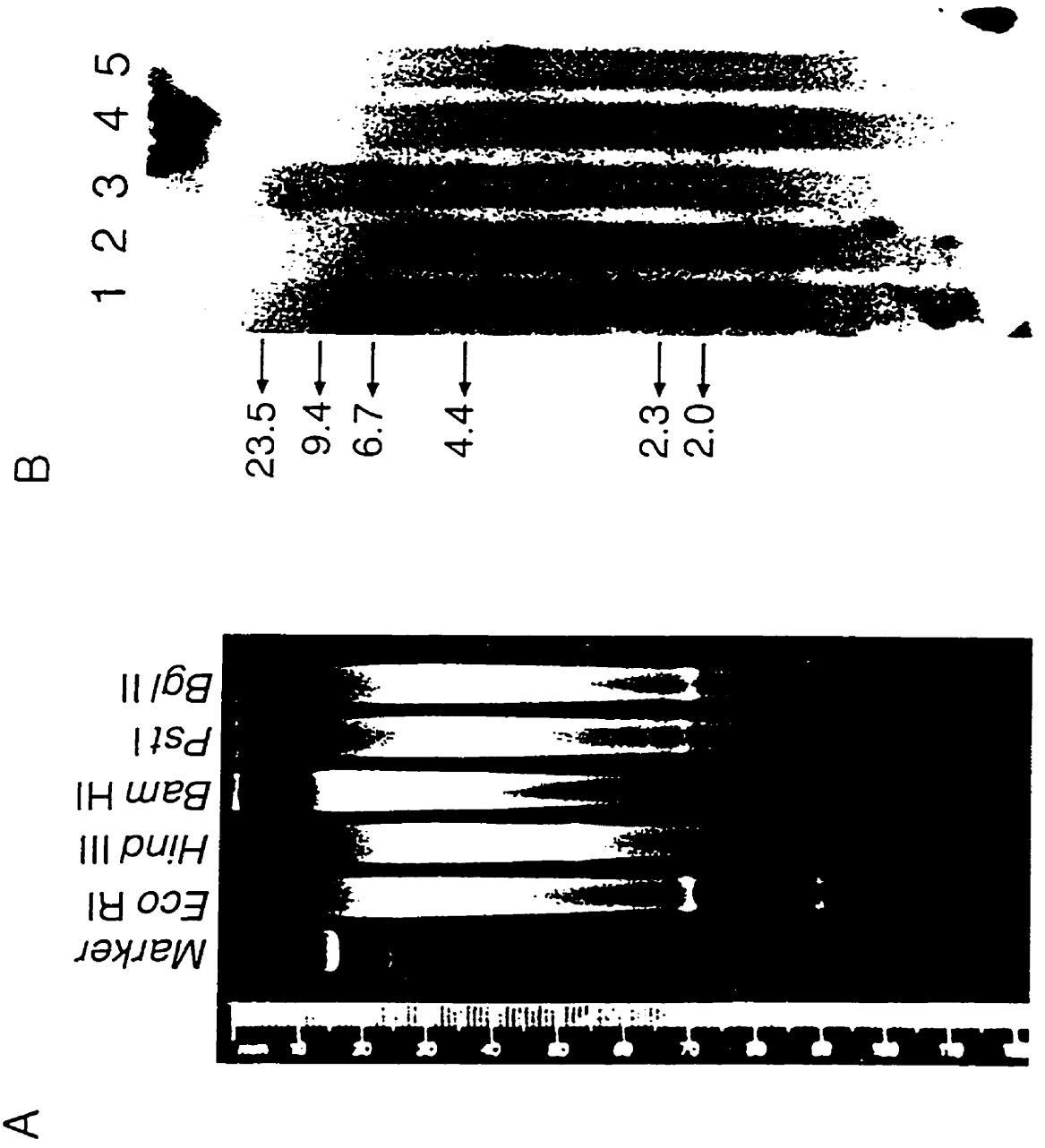
A
Marker
Eco RI
Hind III
Bam HI
Pst I
Bgl II
B
1 2 3 4 5
23.5
9.4
6.7
4.4
2.3
2.0

Figure 11

Figure 12. The putative rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase cDNA codes for a protein recognized by anti-human platelet CD 36. **Panel A.** Immunoblot analysis of CD36 in extracts of *E. coli* cells transformed with pTricHis containing cDNA for rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase inserted into orf B site. Lanes 1-5 correspond to the solubilized cell extracts prepared (0 hr, 1 hr, 2 hr, 3 hr, and 16 hr) after induction with IPTG. Immunoblots were developed with anti-human platelet CD36 monoclonal antibody used as a primary antibody. **Panel B.** Immunoblot analysis of CD36 in total cell extracts of *E. coli* cells transformed with vector only [pTricHis (orf B)]. Lane assignments are identical to those in panel A. Immunoblots were developed with anti-human platelet CD36 monoclonal antibody used as a primary antibody.

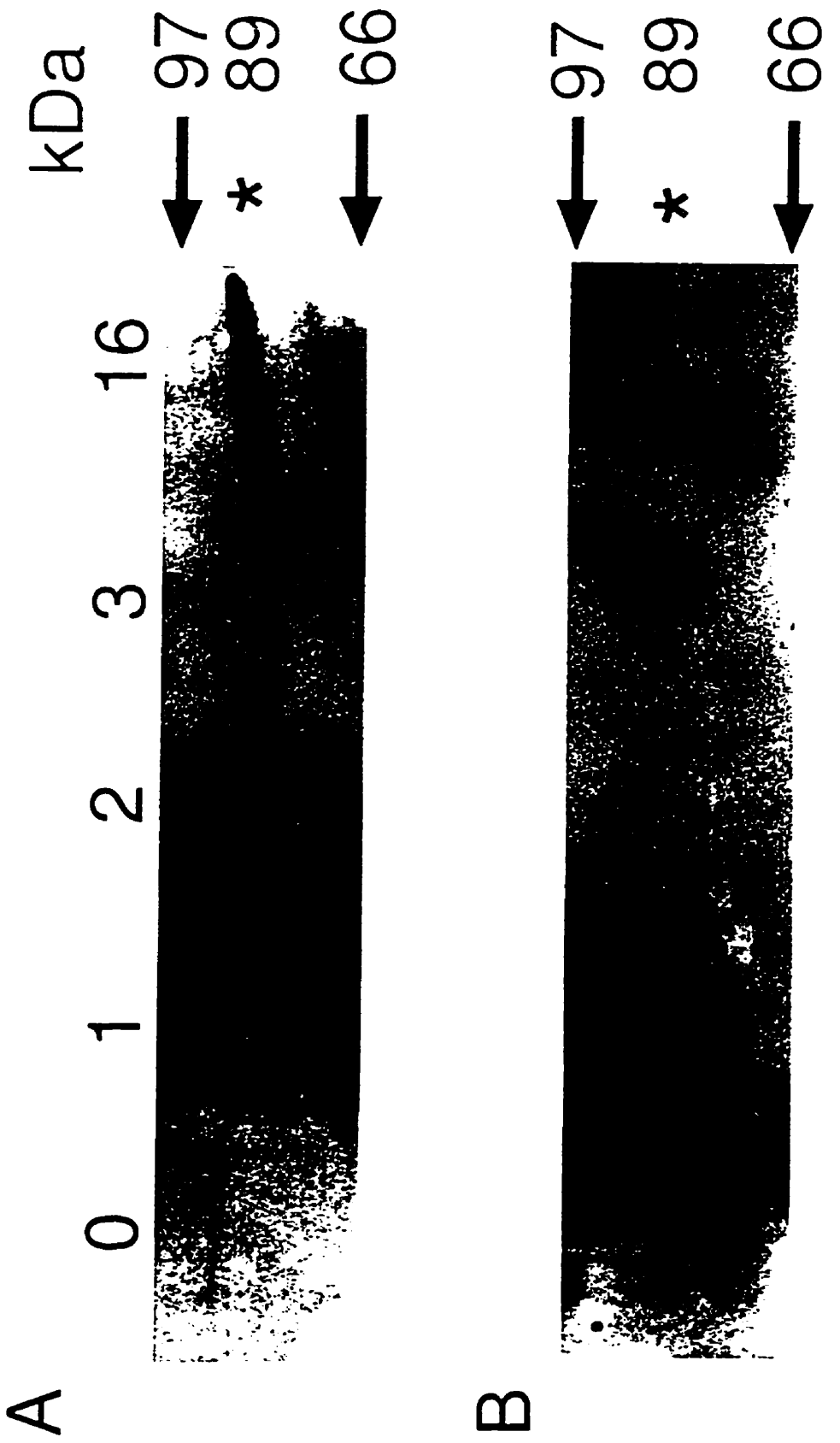

Figure 12

3. Preparation and localization of an antiserum against rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase

A. Characterization of anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

An antiserum was raised against purified rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ATPase and its sensitivity and potency were tested by immunoblotting. A 1:1300 antiserum dilution was capable of detecting antigen concentrations as low as 13 ng when tested against a range of antigen concentrations (1 ng to 1 μg/lane). When different dilutions of the antiserum were examined (1:1300-1:50,000), a 1:20,000 dilution was sufficient for detecting 130 ng of antigen; pre-immune serum did not react with the antigen under any conditions (data not shown).

The specificity of anti-rat Ca^{2+}/Mg^{2+} ATPase serum was tested against the purified Ca^{2+}/Mg^{2+} ATPase as well as other Ca^{2+} ATPases from sarcolemma and sarcoplasmic reticulum. Figure 13 shows reactivity of the immune serum (1:1300) against purified and membrane proteins; it clearly detects the pure antigen (2-3 μg) (Fig. 13, Lane 4) as well as a band with identical mobility in the sarcolemmal preparation (5 μg) (Fig. 13, Lane 2), but does not crossreact with rat sarcoplasmic reticulum Ca^{2+} ATPase (5 μg) (Fig. 13, Lane 3) or sarcolemmal Ca^{2+} ATPase-pump (5 μg). Lane 1 is purified ATPase stained by silver staining.

B. Immunofluorescent staining of cardiac tissue with anti-Ca^{2+}/Mg^{2+} ecto-ATPase IgG

Frozen heart tissue sections were examined for immunostaining with the immune as

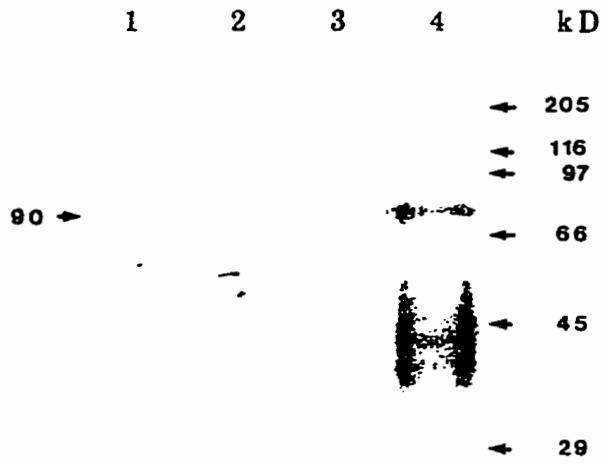

Figure 13. Immunoblots of rat cardiac Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase with antibodies. Lane 1: purified rat cardiac sarcolemmal ecto-ATPase protein, 2 μg (silver stained); Lane 2: cardiac sarcolemma, 5 μg; Lane 3: cardiac sarcoplasmic reticulum, 5 μg; Lane 4: purified cardiac Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase, 2 μg. All of the samples were individually treated with SDS-PAGE buffer and loaded on the gel. Anti-rat cardiac sarcolemma Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase antibody was used at a dilution of 1:5000.

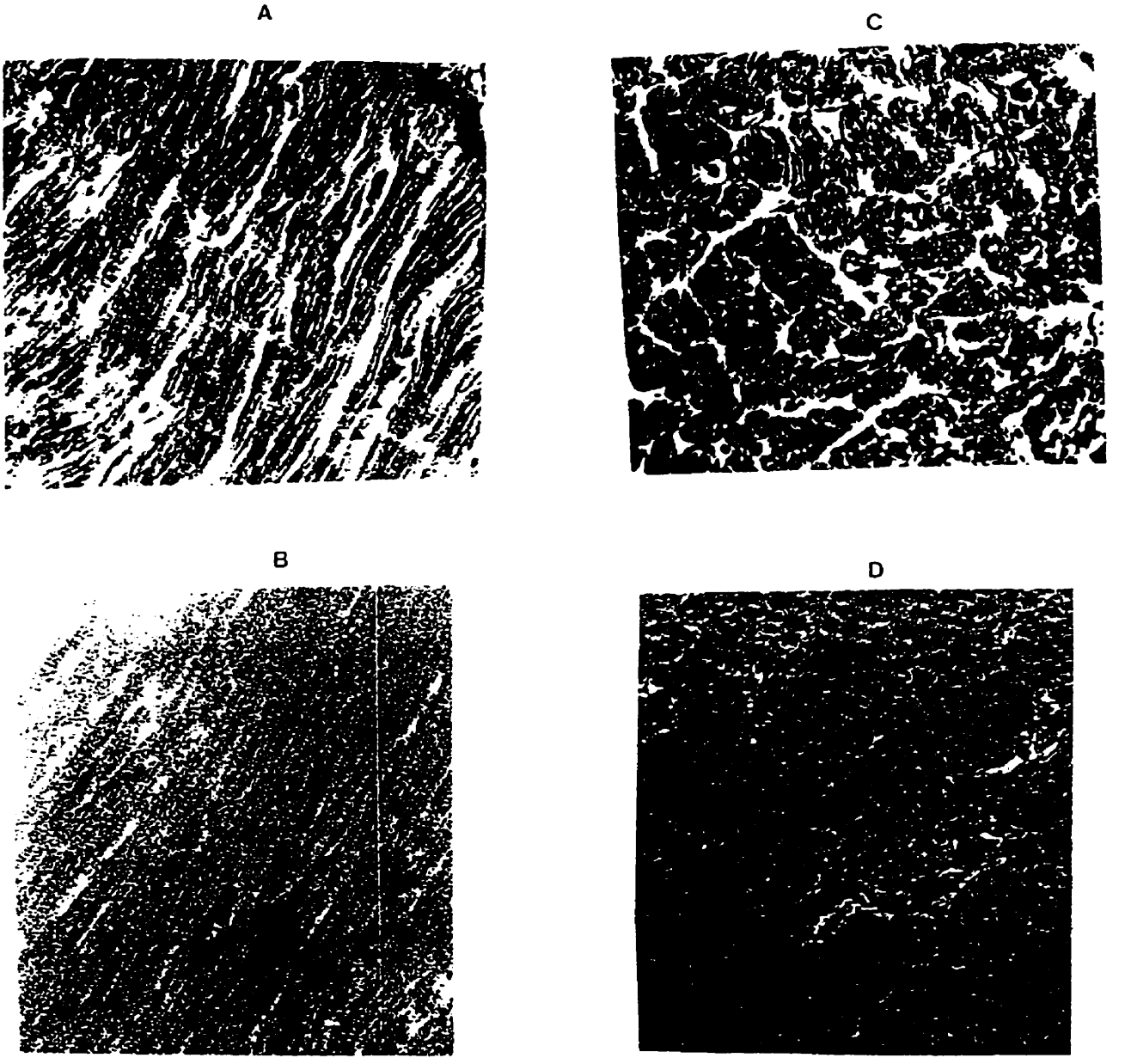

Figure 14. Micrographs of transverse and longitudinal cardiac section. **A)** transverse section incubated with immune anti-Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase antiserum (arrow heads show staining on the plasma membrane of cardiac section). **B)** incubated with pre-immune serum, indicating absence of staining in the plasma membrane. **C)** and **D)** are longitudinal sections incubated with C) immune and **D)** pre-immune serum.

well as pre-immune sera. As seen in Figure 14, staining was seen mainly around myocytes in apparent association with the plasma membranes of these cells. Staining was also evident around non-muscle cells, presumably capillaries or fibroblasts. Some staining was also seen in the myocyte cytoplasm. As this cytoplasmic staining was also present in sections stained with pre-immune serum, it was considered to represent a non-specific reaction, unlike the strong pericellular staining.

C. Antigen specificity of anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase serum

To demonstrate the specificity of anti-rat cardiac Ca^{2+}/Mg^{2+} ATPase serum, an immunoprecipitation assay was performed. In assay medium containing the purified Ca^{2+}/Mg^{2+} ATPase and the immunoglobulin G prepared from immune serum (anti-rat Ca^{2+}/Mg^{2+} ATPase) were incubated in the presence of protein A sepharose. After incubation, the supernatant was added to the tissue sections. Subsequent immunohistochemical localization results show that the (pre-absorbed) reaction supernatant did not detect the ecto-ATPase molecule (Fig. 15).

D. Immunofluorescent staining of cultured cardiomyocytes with anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase IgG

Cultured neonatal myocytes were stained with the immune serum (Fig. 16A). These were also simultaneously stained for vimentin, an intermediate filament protein, present in immature (but not adult) myocytes and non-muscle cells (Fig. 16B). Anti-vimentin staining is myofibrillar in myocytes and helps identify these cells. As seen in Figure 16A, staining for the Ca^{2+}/Mg^{2+} ATPase was localized around cardiomyocytes in areas of cell-substrate or

A

B

Figure 15. Micrographs of heart tissue sections incubated with **A)** absorbed immune anti-Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase antiserum and **B)** incubated with non-absorbed immune serum.

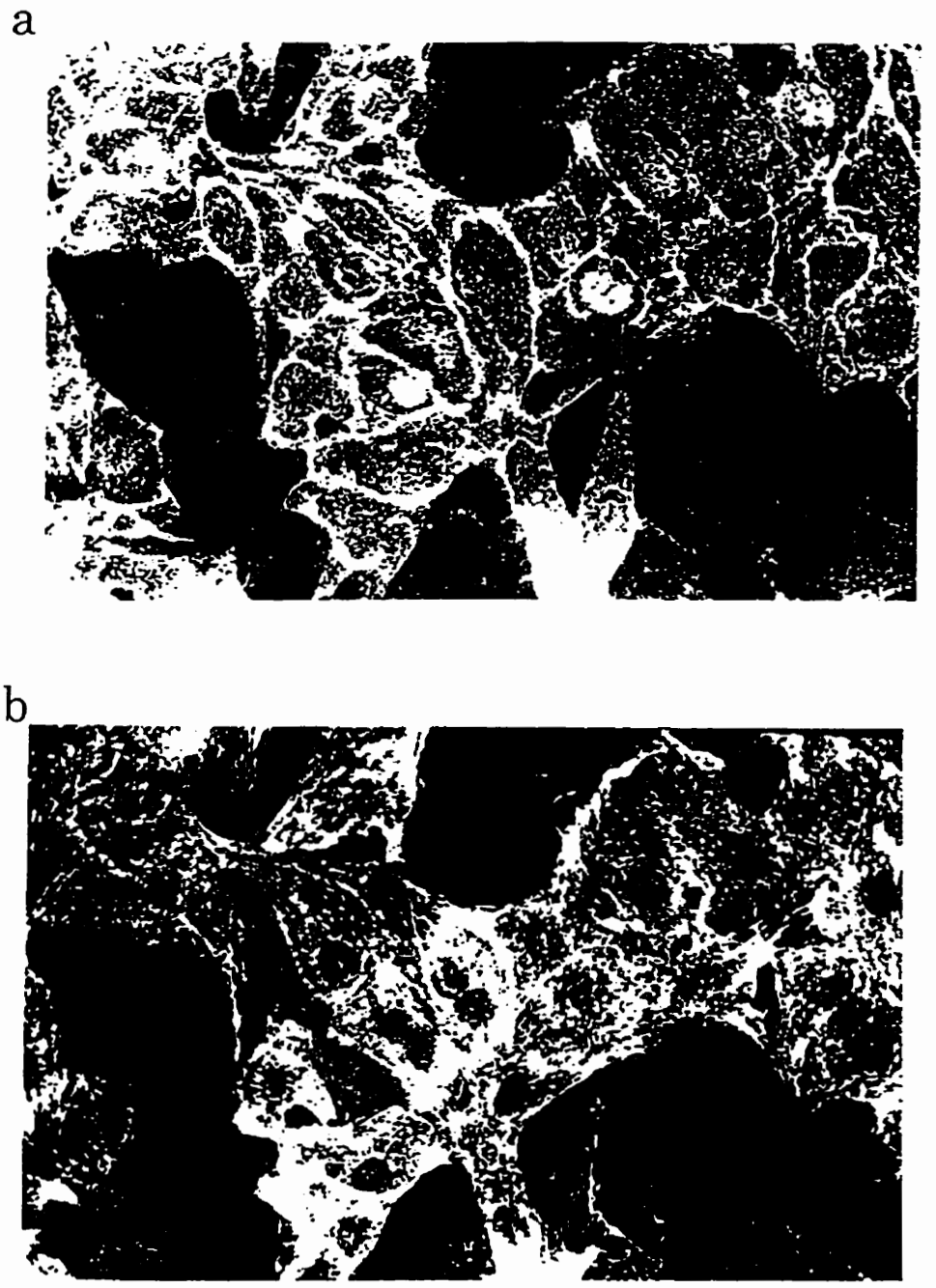

Figure 16. Micrographs of cultured cardiocytes incubated with immune serum. **Panel A.** indicates the surface localization of the Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase; **Panel B.** double fluorescence labelling for myosin.

cell-cell contact.

E. Tissue specificity of anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

The specificity of anti-rat cardiac Ca^{2+}/Mg^{2+} ATPase serum was also analyzed by staining different tissue sections (brain, kidney, liver and skeletal muscle) with immune serum. These data are shown in Figures 17A-D. All tissues were also stained with the pre-immune serum (data not shown). Staining was strong and pericellular in skeletal myofibers and interstitial cells.

F. Immunocrossreactivity of anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase with Ca^{2+}-pump ATPases

To identify the crossreactivity of rat cardiac Ca^{2+}/Mg^{2+} ATPase with other ion transporting ATPases (Ca^{2+}-pump ATPase of sarcolemma and sarcoplasmic reticulum), immunoblotting was carried out with antibodies directed against the sarcolemmal and sarcoplasmic reticulum Ca^{2+}-pump ATPase. In Western immunoblots, the anti-human plasma membrane monoclonal antibody did not crossreact with purified rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ATPase (Fig. 18A, Lane 2) whereas this monoclonal antibody recognized the Ca^{2+}-pump ATPase of sarcolemma ~140 kDa and its proteolytic breakdown product ~90 kDa (Fig. 18A, Lane 1). Anti-rat SR calcium ATPase antibody directed against rat SR Ca^{2+} ATPase did not crossreact with the purified rat cardiac Ca^{2+}/Mg^{2+} ATPase (Fig. 18A, Lane 2) whereas this polyclonal antiserum recognized the SR Ca^{2+}-pump ATPase in the SR preparation separated on the SDS-PAGE (Fig. 18B, Lane 1).

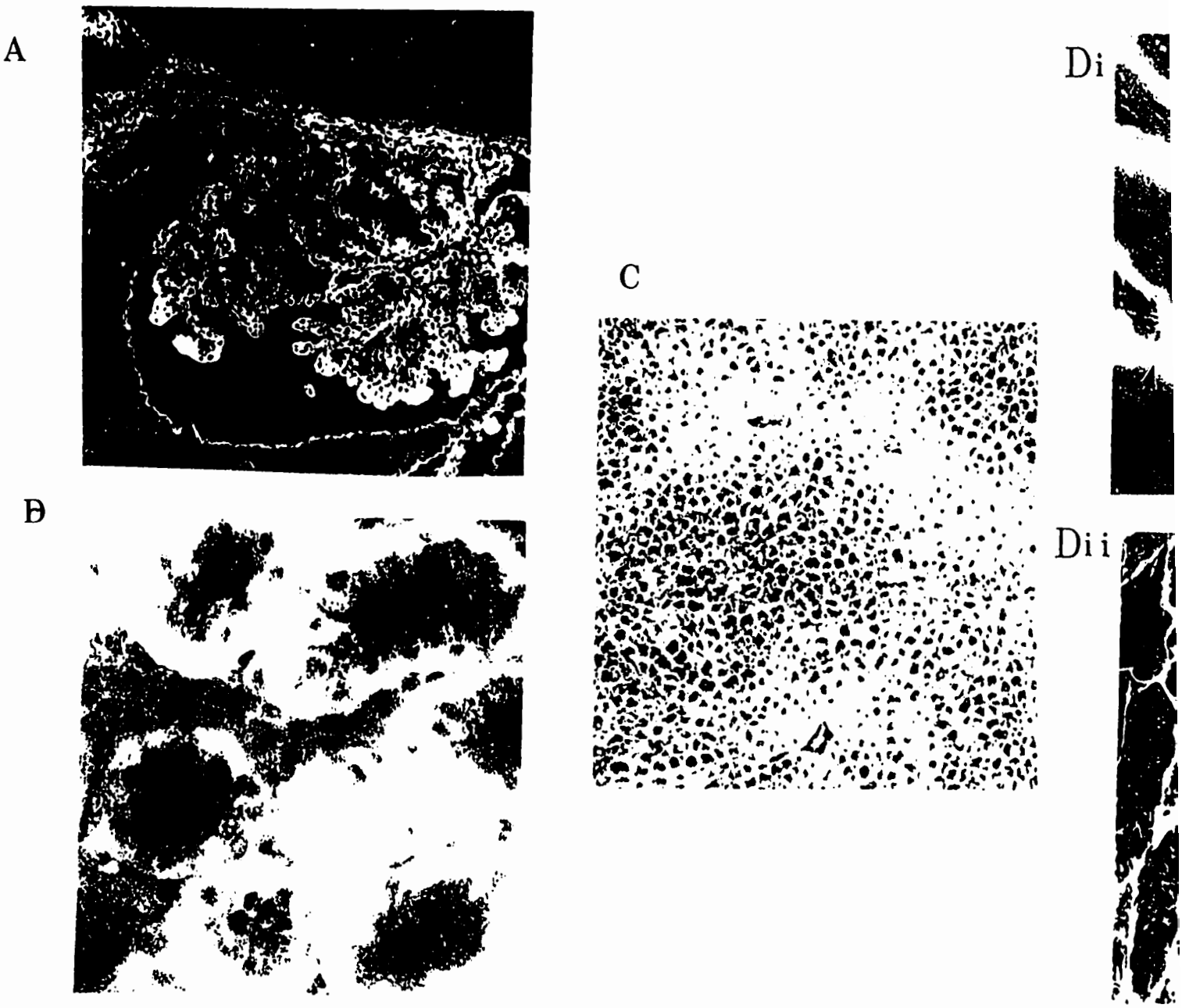

Figure 17. Micrographs of tissue sections. A) brain; B) kidney; C) liver and D) muscle (i. transverse and ii. longitudinal sections) incubated with immune anti-rat C ATPase/ecto-ATPase antiserum.

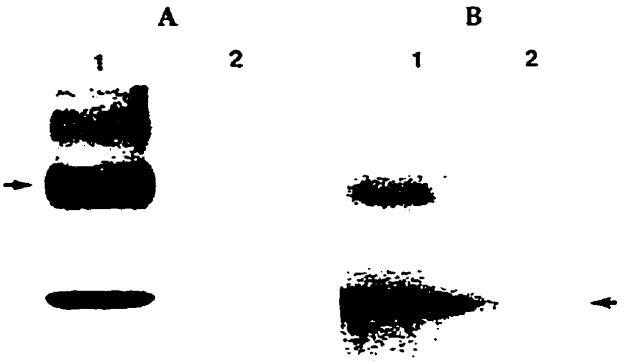

Figure 18. Immunoblots of cardiac Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase crossreactivity with anti-human erythrocyte plasma membrane calcium-pump (Affinity Bioreagents); #MA3-914 monoclonal (mouse) anti-PMCA ATPase antibody (IgG2a Clone 5F10; Neshanic Station, NJ) and anti-rat SR Ca^{2+} ATPase serum 1:1000 dilution were used. **Panel A.** Lane 1: 5 µg of rat cardiac sarcolemma; Lane 2: 2 µg of purified rat cardiac ecto-ATPase incubated with anti-PMCA antibody (1:1000 dilution). **Panel B.** Lane 1: 5 µg of rat cardiac sarcoplasmic reticulum; Lane 2: 2 µg of purified rat cardiac ecto-ATPase incubated with anti-rat SR Ca^{2+} ATPase antibody (1:1000 dilution).

G. Inhibition of Ca^{2+}/Mg^{2+} ATPase activity by anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase IgG

The ability of the anti-rat cardiac Ca^{2+}/Mg^{2+} ATPase antiserum to recognize the antigen (rat cardiac Ca^{2+}/Mg^{2+} ATPase) was analyzed by indirect immunoprecipitation in the presence of purified Ca^{2+}/Mg^{2+} ecto-ATPase and purified immunoglobulin (IgG) from the immune or pre-immune serum. The purified IgG fraction of anti-rat cardiac Ca^{2+}/Mg^{2+} ATPase antiserum inhibited the Ca^{2+}/Mg^{2+} ATPase activity in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} in immunoprecipitated pellet suspension as shown (Figs. 19 A and B) whereas the Ca^{2+}/Mg^{2+} ATPase activity remained detectable in the supernatant of the reaction medium in the presence of the 4 mM Ca^{2+} (Fig. 19C) or 4 mM Mg^{2+} (Fig. 19D). The results indicate that the anti-rat Ca^{2+}/Mg^{2+} ATPase antiserum is specific and recognizes the antigen (rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ATPase).

4. Cell adhesion properties of the Ca^{2+}/Mg^{2+} ecto-ATPase

A. Cell adhesion bio-assay

In order to investigate whether cardiac Ca^{2+}/Mg^{2+} ecto-ATPase has any role in cell adhesion, we examined the effect of the purified anti-Ca^{2+}/Mg^{2+} ecto-ATPase IgG fraction, obtained from the immune and pre-immune sera, on neonatal cardiomyocyte attachment to the substratum. In the first set of experiments, cardiomyocytes were allowed to attach to the substratum for 4 hr and then incubated with pre-immune or immune IgG fraction for 48 hr. The results in Fig. 20 (Panels A and E) show that cardiomyocytes in the presence of pre-immune IgG remained attached to the substratum; these were flattened and spread in

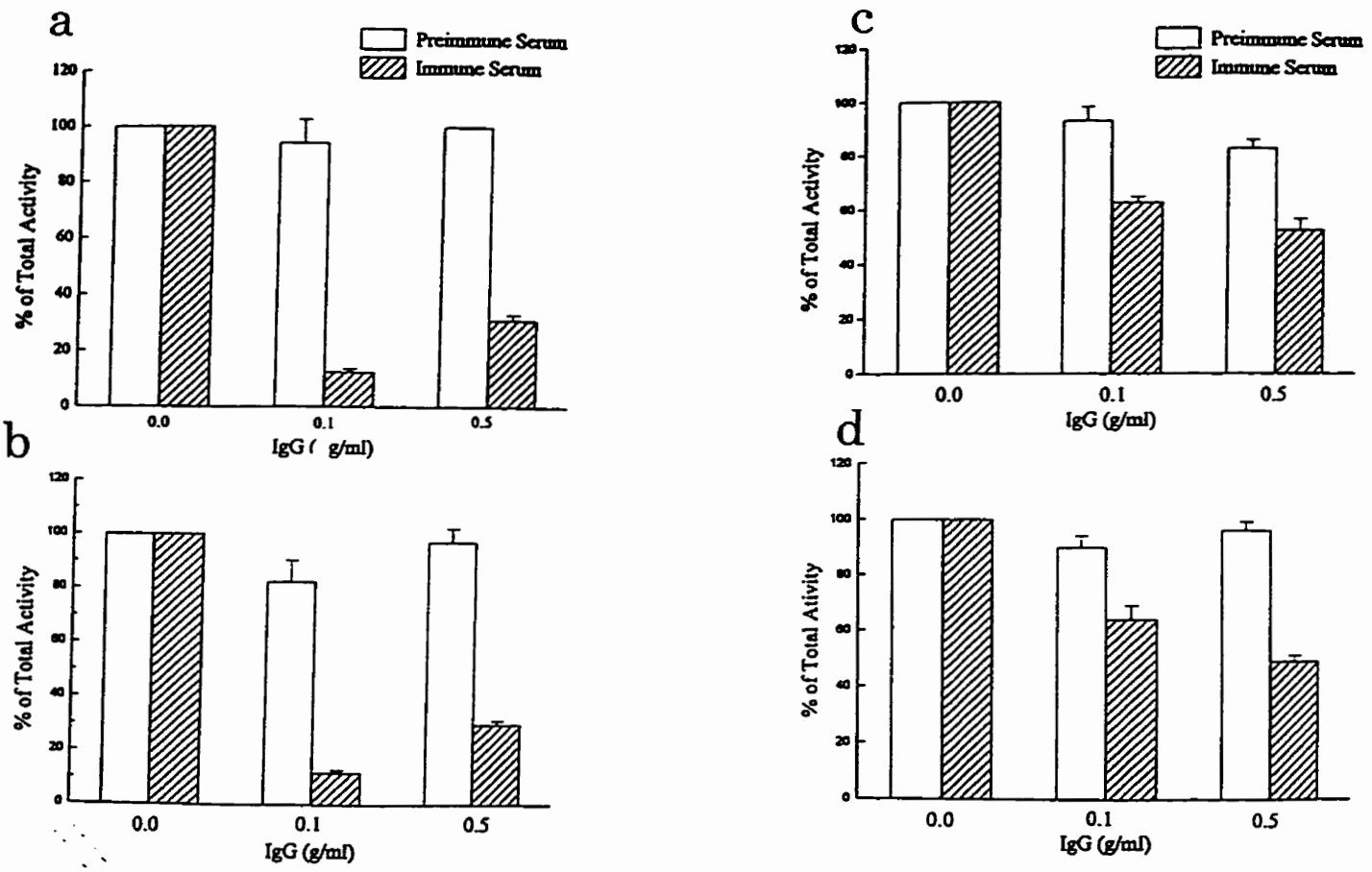

Figure 19. Immunoreactivity of antibodies against cardiac Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase. An indirect immunoprecipitation of purified Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase by IgG fraction of anti-rat cardiac ecto-ATPase antiserum. **A)** 4 mM Ca^{2+}, pellet suspension; **B)** 4 mM Mg^{2+}, pellet suspension; **C)** 4 mM Ca^{2+}, supernatant; and **D)** 4 mM Mg^{2+}, supernatant. 1 µg of purified cardiac Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase was added to the 0.4 µg IgG fraction of immune or pre-immune antiserum in the presence of protein A sepharose 4B in 50% suspension in TBS incubated at 4°C overnight with constant mixing. The sample was centrifuged in an Eppendorf microfuge for 15 sec. The pellet was suspended in 100 µl of 50 mM Tris-Cl, pH 7.5; in both pellet suspension and supernatant, the Ca^{2+}/Mg^{2+} ATPase activity was carried out in the presence of 4 mM Ca^{2+} or 4 mM Mg^{2+} as described by Zhao and Dhalla (1991). Assay was performed in duplicate.

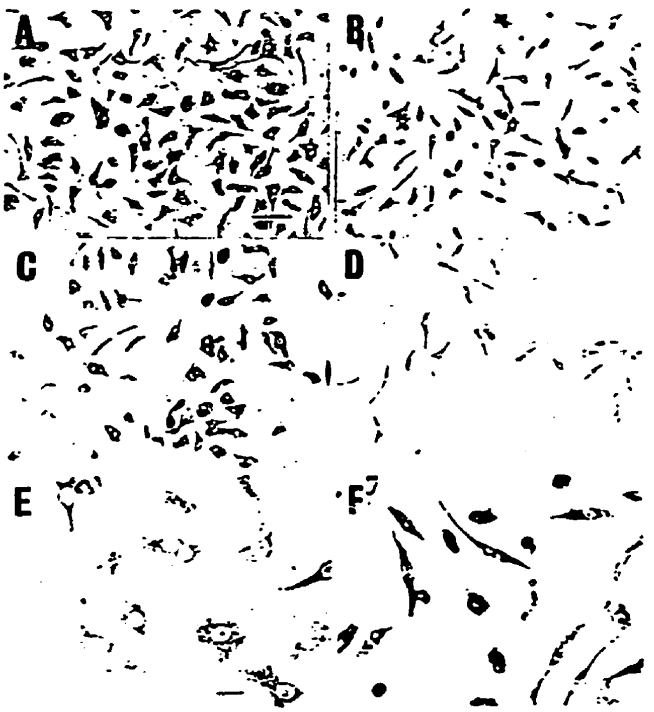

Figure 20. Effect of anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase IgG fraction (100 μg/ml) on morphology and adhesion of cardiomyocyte culture. **Panels A and E** show cells which were allowed to attach for 4 hr and then incubated with pre-immune IgG whereas cultures in **Panels B and F** were incubated with anti-ecto-ATPase IgG for 48 hr. The cells in **Panels C and D** were incubated from the beginning with pre-immune and immune IgG for 48 hr, respectively. Bar in Panel A = 100 μm (magnification for Panels B, C and D was the same). Bar in Panel E = 20 μm (magnification for panel F was the same). All cultures were processed for PAS staining (Sigma kit).

several directions. On the other hand, cardiomyocytes (Fig. 20 - panels B and F) in the presence of immune IgG fraction (anti-cardiac Ca^{2+}/Mg^{2+} ecto-ATPase) became spindle-shaped and elongated (about 55%) or round-shaped and small (about 45%). Identical results to those seen with pre-immune and immune IgG fractions were obtained when pre-attached cardiomyocytes were incubated with 0.5% pre-immune and immune sera (data not shown). The phase II set of experiments was carried out by incubating cardiomyocytes (without prior attachment to substratum) with pre-immune or immune IgG fraction for 48 hr and the results are shown in panels C and D of Fig. 20. Incubation of cardiomyocytes with immune IgG decreased the cell attachment to substratum by about 60% in comparison to that with pre-immune IgG; these cells also became spindle-shaped. Since fewer spindle-shaped cells were consistently observed in the presence of immune (compared to pre-immune) IgG, it is suggested that Ca^{2+}/Mg^{2+} ecto-ATPase may be important for the initial interaction of myocytes with their substratum and may provide sites for their adhesion (Kannan et al., 1997).

B. Immunocrossreactivity of rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase with anti-human CD36

Immunoblot analysis was conducted to determine the cross reactivity of purified Ca^{2+}/Mg^{2+} ecto-ATPase protein with mouse anti-CD36 monoclonal antibodies. The results indicated that the anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase serum recognized the purified Ca^{2+}/Mg^{2+} ecto-ATPase molecule and the human CD36 molecule present in the Jurkat cell lysate (Fig. 21, panel A); the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase showed crossreactivity with

anti-CD36 (monoclonal antibodies) (Fig. 21, panel B). The results suggest that both human CD36 molecule and rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase share immunological crossreactivity (Kannan et al., 1997).

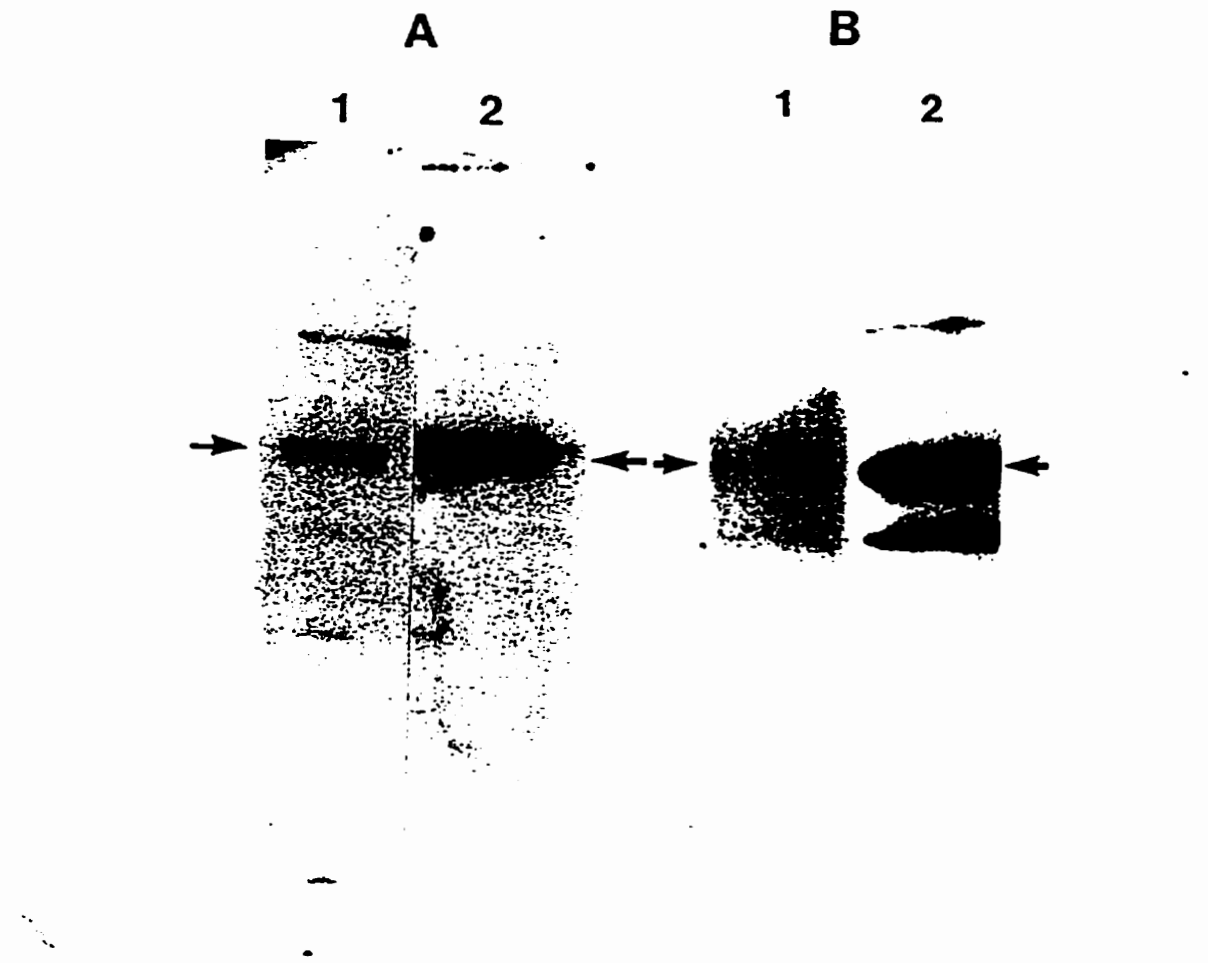

Figure 21. Western immunoblots. **Panel A.** Lane 1: 4 l (1.6 g) of Jurkat cell lysate (Acute T-cell leukemia cell line Cat # J 14900); Lane 2: 100 ng of purified cardiac Ca^{2+}/Mg^{2+} ecto-ATPase. Samples were individually treated with the SDS-PAGE loading buffer, boiled for 90 sec and loaded on the gel. The blot was probed with the anti-rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase serum at 1:1000 dilution. A band at ~88 kD in Lane 1 indicates CD36 molecule present in Jurkat cell lysate and a band at ~90 kD in Lane 2 indicates that the Ca^{2+}/Mg^{2+} ecto-ATPase protein was recognized by anti-rat Ca^{2+}/Mg^{2+} ecto-ATPase cardiac serum. **Panel B.** Western immunoblots of the Ca^{2+}/Mg^{2+} ecto-ATPase probed with anti-human CD36 (1:1000) (monoclonal antibodies; Transduction Laboratories, Glentech, Inc., Kentucky, USA). Lane 1: 5 l of Jurkat cell lysate (Acute T-cell leukemia cell line Cat # J 14900); Lane 2: 2 g of purified cardiac Ca^{2+}/Mg^{2+} ecto-ATPase; samples were treated as above. A band at ~88 kD band in Lane 1 indicates CD36 molecule present in the Jurkat cell lysate and a band at ~90 kD in Lane 2 indicates that the Ca^{2+}/Mg^{2+} ecto-ATPase protein is recognized by anti-human CD36 serum.

V. DISCUSSION

1. Structural properties of the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase

Although Ca^{2+}/Mg^{2+} ecto-ATPase has been purified and characterized from different tissue sources, the precise molecular structural properties remain unknown (Dhalla and Zhao, 1989; Plesner, 1995). Earlier we have shown that the rat Ca^{2+}/Mg^{2+} ecto-ATPase has cell adhesion properties and thus was named as Myoglein (Kannan et al., 1997). The isoelectric point at 5.7 of the rat Ca^{2+}/Mg^{2+} ecto-ATPase indicates that the enzyme is acidic in nature. Electroelution, SDS-PAGE and two dimensional electrophoresis showed that the purified protein is composed of at least two polypeptide components with MW of ~ 90 and 85 kD. The rabbit skeletal muscle T-tubules, Ca^{2+}/Mg^{2+} ecto-ATPase was shown to be composed of 2 polypeptides with MW of 107 and 30 kD (Hidalgo et al., 1983) while the rabbit skeletal muscle T-tubular Mg^{2+} ATPase was found as a monomer with MW of 105 kD (Kirley, 1988). Sheep kidney medulla plasma membrane Ca^{2+}/Mg^{2+} ecto-ATPase was resolved as two polypeptide components with MW 150 and 77 kD (Gantzer and Grisham, 1979). Human oat cell Ca^{2+}/Mg^{2+} ecto-ATPase contained only a 30 kD subunit (Knowles and Leng, 1983). Tuana and Dhalla (1985), demonstrated that Ca^{2+}/Mg^{2+} ecto-ATPase purified from the cardiac heavy sarcolemmal membrane was composed of two polypeptide components of MW 55 and 12 kD. These observations are consistent with the view that Ca^{2+}/Mg^{2+} ecto-ATPase may exist in multiple isoforms.

Mass spectroscopic analysis of purified Ca^{2+}/Mg^{2+} ecto-ATPase with three matrix supports such as [2-(4-hydroxy phenylaxol)]-benzoic acid (HABA), 3,5-dimethoxy-4-

hydroxy cinnamic acid (SINAPINIC), and alpha-cyano-4-hydroxy cinnamic acid (ALPHA) showed multicomponent species of different molecular masses ranging from 1 to 5 kD. Such a pattern is indicative of microheterogeneity in the structure of the enzyme. Since pre-treatment of Ca^{2+}/Mg^{2+} ecto-ATPase with DL-dithiothreitol did not change the mass spectroscopic pattern, the microheterogeneity of cardiac Ca^{2+}/Mg^{2+} ecto-ATPase may be attributed to any of the posttranslational modifications (eg. glycosylation or acetylation or myrstilation). In fact, this protein has been shown to be a glycoprotein by periodic Schiffs staining (Zhao et al., 1991).

2. Molecular characterization of rat cardiac SL Ca^{2+}/Mg^{2+} ecto-ATPase

This part of the study reports that rat cardiac SL Ca^{2+}/Mg^{2+} ecto-ATPase may either contain a fragment with cell adhesion properties or is tightly associated with a protein which is homologous to the adhesion molecule CD36. There are two lines of evidence to support this conclusion. First, by utilizing oligomers based on the amino acid sequence of the rat cardiac SL Ca^{2+}/Mg^{2+} ecto-ATPase, a cDNA fragment having nucleotide sequence homologous with CD36 and other cell adhesion molecules was cloned. Second, expression of pND2.1 produced a protein that was recognized by an antibody specific for CD36, but not by antibody for rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase.

It has previously been suggested that E-type ATPase activity may be tightly bound to proteins having adhesion characteristics. This conclusion was based on studies conducted on both ecto-ATPases and cell adhesion molecules. It was observed that the sequence for the ecto-ATPase purified from rat liver is homologous to the human biliary glycoprotein I

(Lin and Guidotti, 1989). Furthermore, it was noted that the liver ecto-ATPase cDNA sequence contains neither of the walker motives [**I**: $GX_1X_2X_3X_4$-GK or **II**: R/K-$X_1X_2X_3$-L- (where X = hydrophobic residues)] representative of the ATP binding region of ion transporting ATPases (Lin and Guidotti, 1989). While the absence of a walker motif may indicate this protein is not capable of ATP hydrolysis, this feature is characteristic for ion motive ATPases forming phosphoprotein intermediates (Shull and Greeb, 1988) and the rat liver plasma membrane high affinity Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase is insensitive to vanadate. It is implied that aspartyl phosphate intermediate formation is not required for ATP hydrolysis by rat liver ecto-ATPase (Lin, 1985). Expression of the rat liver ecto-ATPase cDNA, however, demonstrated that this clone coded for a protein in *E. coli* which had the properties of a cell adhesion protein and simultaneously lacked ATPase activity (Cheung et al., 1993). It may nevertheless be possible that this particular isoform of liver ecto-ATPase has an activity too low for the detection due to inefficient folding in the bacterial cell.

Similar to the rat liver enzyme, cloning of the chicken gizzard smooth muscle ecto-ATPase produced a cDNA with a sequence that has considerable sequence homology with mouse and human CD39 (Kirley, 1997). Interestingly, CD39 is a cell adhesion protein that has been found to have an ecto (Ca^{2+}/Mg^{2+}) apyrase activity capable of hydrolysing both ATP and ADP (Wang and Guidotti, 1996; Kirley, 1997; Beaudoin et al., 1997). Expression of the chicken gizzard smooth muscle ecto ATPase cDNA has yet to be reported and until the expression product has been functionally characterized, identification of the cDNA as

an ecto-ATPase remains impossible.

While there is no direct or indirect evidence to prove or disprove that an ecto-ATPase/E-type ATPase activity is associated with adhesion molecules, it is intriguing to note that β_2-subunit of the sodium-pump (Na^+/K^+ ATPase) possesses homology with cell adhesion molecule of glia (Gloor et al., 1990). Similarly, the ecto 5' nucleotidase (hydrolyzes AMP to adenosine)/CD73 [E.C.3.1.3.5] has also been demonstrated to harbor and/or function as an adhesion molecule (Airas and Jalkanen, 1996). Thus, there is considerable evidence to indicate E-type ATPases are directly or indirectly associated with the cell adhesion proteins.

As was noted for CD39, the converse relationship is also possible. Proteins involved in adhesion (eg. NCAM, C-CAM, CD36, CD39) may possess an inherent extracellular ATP-binding domain capable of hydrolyzing this nucleoside triphosphate. Adhesion molecules exhibiting this property are insensitive to inhibitors of P-type, V-type and F-type ATPases (Plesner et al., 1997). While evidence for the close relationship between E-type ATPases and cell adhesion molecules was documented by several research groups (Lin and Guidotti, 1989; Cunningham et al., 1993; Cheung et al., 1993; Dzhandhugazyan and Bock, 1993; 1997a,b; Knowles, 1995; Kirley, 1997; Kannan et al., 1997), an accurate description of how these distinct activities are coupled has yet to be attained. Resolution of this issue remains controversial as demonstrated by the divergent conclusions reached with respect to NCAM: Stout et al. (1994) demonstrated NCAM can be physically separated from an ecto-Mg^{2+} ATPase activity by anion exchange chromatography, while Dzhanzhygazyan and

Bock (1997b) showed that NCAM can be affinity labelled with an ATP analogue. In the case of the cardiac Ca^{2+}/Mg^{2+} ecto-ATPase, however, as was noted for the rat liver plasma membrane ecto-ATPase (Lin and Guidotti, 1989), isolation of a cDNA clone based on the sequence obtained from the purified enzyme provided a gene that was highly homologous to the adhesion protein CD36. Moreover, expression of the cDNA yielded a protein that was recognized by an anti-CD36 antibody but not by an anti-Ca^{2+}/Mg^{2+} ecto-ATPase antibody (data not shown). These results suggest that the cardiac Ca^{2+}/Mg^{2+} ecto-ATPase consists of distinct two components, a fragment which hydrolyzes ATP and a fragment which functions in cell adhesion.

In conclusion, our objective was to isolate a full-length cDNA clone for the rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase; however, in this process, we observed that a component of the E-type ATPase is a fragment identified as an adhesion molecule, CD36 (Kannan et al., 1997). Furthermore, these data indicate that the component exhibiting Ca^{2+}/Mg^{2+} ecto-ATPase activity is immunologically distinct from the cell adhesion molecule. It is possible, however that this close association between Ca^{2+}/Mg^{2+} ecto-ATPase and CD36 is an important factor in the regulation of cell-cell adhesion processes in the heart (Dzhandzugazyan and Bock, 1997a; Stout and Kirley, 1996; Kirley, 1997; Lewis Carl and Kirley, 1997).

3. Immunochemical characterization of rat cardiac SL Ca^{2+}/Mg^{2+} ecto-ATPase

By utilizing an antiserum against purified rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase, the ecto-ATPase molecule was localized on the cardiac tissue and cultured

cardiomyocyte plasma membrane and demonstrated that it is an antigen specific antiserum. There are two line of evidences to support this conclusion. First an immunoblot analysis showed a 90 kD band in the purified SL membrane. As well, purified Ca^{2+}/Mg^{2+} ecto-ATPase and antigen were recognized by the anti-Ca^{2+}/Mg^{2+} ATPase antibody and purified immunoglobulin. Secondly, using polyclonal antibodies against purified Ca^{2+}/Mg^{2+} ATPase, immunofluorescent staining was observed on the plasma membranes of heart, kidney, skeletal muscle, liver and brain tissue sections as well as cultured cardiocytes. There are some reports on the localization of the Ca^{2+}/Mg^{2+} ATPase (ecto-ATPase) on the plasma membrane of the liver tissue, but there is no direct evidence to show the ecto (outside) nature of this enzyme. Liver Ca^{2+}/Mg^{2+} ATPase (ecto-ATPase) is considered to be an ecto-enzyme because the nucleotide hydrolyzing site faces the outside of the cell (Lin and Russell, 1988). Two polyclonal antibodies were raised against the purified rat liver Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase and used to determine the surface distribution of the enzyme by immunofluorescence staining. Ca^{2+}/Mg^{2+} ATPase (ecto-ATPase) was shown to be concentrated on the canalicular surface of the hepatocyte (Lin, 1989).

Two distinct forms (long and short) of liver Ca^{2+}/Mg^{2+} ATPase or ecto-ATPase or C-CAM (110 kDa) are localized on the surface of the rat liver canalicular domain. The long isoform of liver ecto-ATPase has been shown to mediate the cell adhesion function. The short form does not contribute to the cell adhesion function (Cheung et al., 1993a; 1993b). Although the liver Ca^{2+}/Mg^{2+} ATPase has been localized on the plasma membrane, it did not crossreact with cardiac Ca^{2+}/Mg^{2+} ATPase. It is possible that the difference in molecular

weight between liver ecto-ATPase (110 kD) and cardiac Ca^{2+}/Mg^{2+} ATPase (180 kD) may account for the lack of crossreactivity. ATPDase, an enzyme shown to hydrolyze the ATP and ADP at equal ratio, is also reported as an ecto enzyme and referred as CD39 (Kaczmarek et al., 1996). An anti-porcine ATPDase antibody has been shown to recognize the ATPDase on the surface of bovine purkinje fibre cells and the coronary vessels of heart ventricle (Beaudoin et al., 1997).

Cytochemical methods demonstrated that Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase reaction product is formed on the cell surface in rat cerebellum glia cells, neuronal cells process (Mughal et al., 1989) and cultured chromaffin cells (Kriho et al., 1990). Furthermore, using rat liver Ca^{2+}/Mg^{2+} ATPase/C-CAM/immune serum, the liver Ca^{2+}/Mg^{2+} ATPase was localized on the brush border membrane of renal cortical membranes. However, no specific staining in the kidney was obtained by using polyclonal anti-peptide antibodies directed against the long isoform (C-CAM 1) of liver ecto-ATPase (Sabolic et al., 1992). By cytochemical methods and cerium precipitation, the Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase and 5'-nucleosidase activity were localized in the caveolae of smooth muscle cells of guinea pig vas deferens and ileum longitudinal muscle strips (Kittel and Bacsy, 1994). Cytochemical distribution of ATP dephosphorylating activity was specifically localized to the transitional zone where the sensory dendrites develop cilia and branch from the outer dendritic segments, olfactory sensilla (aesthetases), of the spiny lobster. From this localization study, it was suggested that ecto-enzymatic activity may be an important means of removing excitatory/inhibitory nucleotides from this region (Glesson et al., 1992).

Immunogold labelling and indirect immunofluorescence labelling show that the brush borders of S1 and S3 segments of the proximal tubules of kidney were stained for Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase. However, the staining was not detected in the basolateral membrane of the proximal tubule. Endothelial layers of both larger vessels and capillaries were positively stained (Sabolic et al., 1992). It has been proposed that the function of the sinusoidal Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase is to regulate the P_2-purinergic receptor/effector system while the canalicular Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase is involved in the mechanism of retrieval of secreted nucleotides (Lin, 1990). Based on the immunochemical similarity between Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase and rat liver cell adhesion molecule, cell-CAM 110, it was concluded that liver Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase is a cell adhesion molecule (Lin et al., 1991). Immunochemical characterization of two isoforms of rat liver ecto-ATPase that showed an immunological and structural identity with a glycoprotein cell adhesion molecule with Mr 105,000 (Lin and Guidotti, 1989). By employing peroxidase-antiperoxide (PAP) and electron microscopic immunogold labelling method, Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase/C-CAM 110 was localized on the luminal and lateral surfaces and the intercellular canaliculi of the acinar cells of both parotid and submandibular glands. The luminal surface of the intercalated ducts was brightly stained whereas those of the striated and excretory ducts were labelled less prominently. From this immunolocalization study, it was suggested that Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase/C-CAM may play a role in the breakdown of ATP stabilization of the microvilli membranes, cell adhesion and involvement in secretory mechanism (Murphy et al., 1994).

The canalicular Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase was localized on the pericanalicular-apical membrane by confocal microscopy and immunofluorescence (Benedetti et al., 1994). In the presence of a millimolar concentration of Ca^{2+} or Mg^{2+}, Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase activity was observed entirely on the external face of the tanycyte plasma membranes. Furthermore, these reactions showed no absolute requirement for Ca^{2+} and were not inhibited by the sulfhydryl inhibitors or calmodulin antagonists. Primary antibody raised against liver Ca^{2+}/Mg^{2+} ATPase stained the choroidal epithelium but not the vessels or ependymyl tanycytes. Thus, it was concluded that it is a Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase, but it is not a sarcolemmal Ca^{2+}-pump (Cardy and Firth, 1993). Pre-oncogenic cultures were devoid of Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase enzyme activity and had smooth surfaces. The transformed cells showed complexities of surface membranes in the form of ruffles and microvilli. Combined cytochemistry and transmission electron microscopy indicated that the Ca^{2+}/Mg^{2+} ATPase/ecto-ATPase was concentrated on the ruffles and microvilli (Karasaki et al., 1977). The ecto-ATPase activity per cell increased as the density of the cell increased. Histochemical investigation revealed that the ecto-ATPase activity was localized intensively on the cell surface cell (Ohnishi and Kimura, 1976).

It has been suggested that there may be two distinct ATPases present in different stages of development. Ecto-ATPase with high substrate affinity is present in the non-oncogenic cell lines and in the sparse cells of hepatoma cell culture while the ecto-ATPase of cell-cell contacts in transformed cells had a low substrate affinity. Also, the transformed cells developed microvilli and/or extensive membrane infoldings at cellular

boundaries where ecto-ATPase is concentrated and the hydrolytic activity per cell was high (Ohnishi and Yamaguchi, 1978; Karasaki et al., 1980). Mokett et al. (1994) showed the presence of ecto-ATPases in the basolateral membrane of hair cells and implicated them in purine turnover. The ecto-ATPase activity was localized on the cell wall of ultrathin sections of cells of streptococcus sanguis. Furthermore, the pattern of ecto-ATPase localization changed with the phase of growth, suggesting that ecto-ATPase may be involved in cell wall development and maintenance (MacFarlane et al., 1994). Based on the results obtained, it is suggested that Ca^{2+}/Mg^{2+} ecto-ATPase is important for the initial interaction of myocytes with their substratum and may provide sites for their adhesion. Due to its muscle origin and cell adhesion properties, the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase has been tentatively suggested as myoglein (myo = muscle; glein = adhesive) (Kannan et al., 1997).

VI. CONCLUSIONS

In this study an analysis of the structure-function relationship of the purified rat cardiac sarcolemmal Ca^{2+}/Mg^{2+} ecto-ATPase was performed. On the basis of the results obtained in this investigation, following conclusions are drawn:

1. The rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase is an acidic protein having two subunits with microheterogeneity in its molecular structure.

2. Molecular characterization indicates that rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase may either contain a fragment with cell adhesion properties or is tightly associated with a protein which is homologous to the adhesion molecule CD36.

3. Immunochemical characterization indicates that the rat cardiac Ca^{2+}/Mg^{2+} ecto-ATPase is present on the surface of the cardiomyocyte and also in other tissues (e.g. brain, kidney, skeletal muscle and liver).

4. The Ca^{2+}/Mg^{2+} ecto-ATPase molecule has been shown to have at least two polypeptide components; one component shares molecular and immunochemical properties similar to the cell adhesion molecule CD36.

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IMAGE EVALUATION
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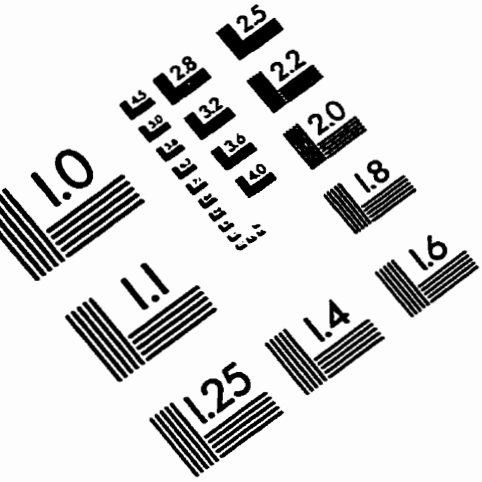

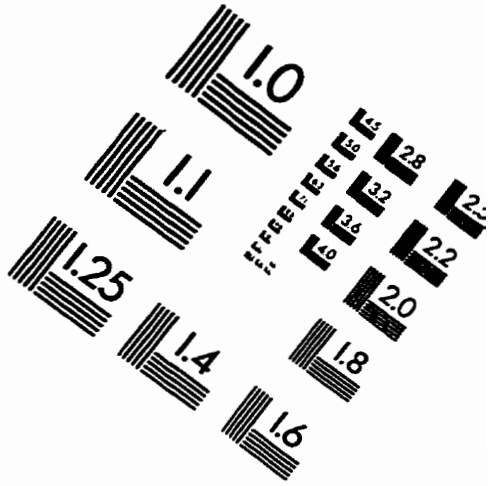

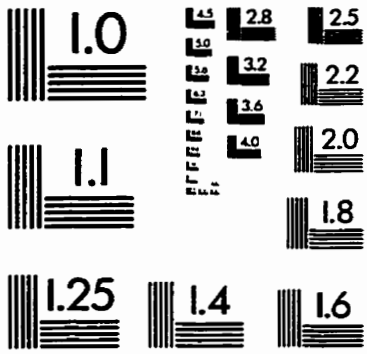

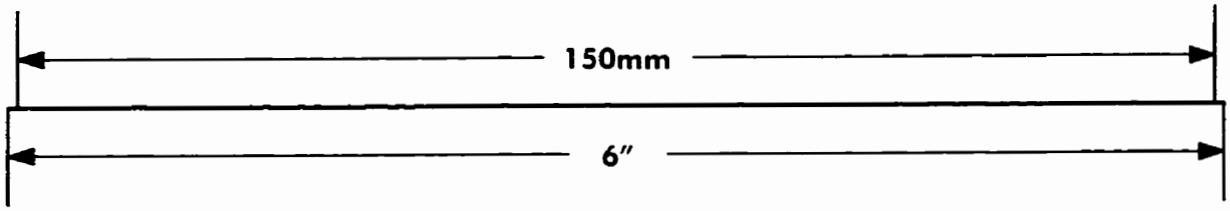

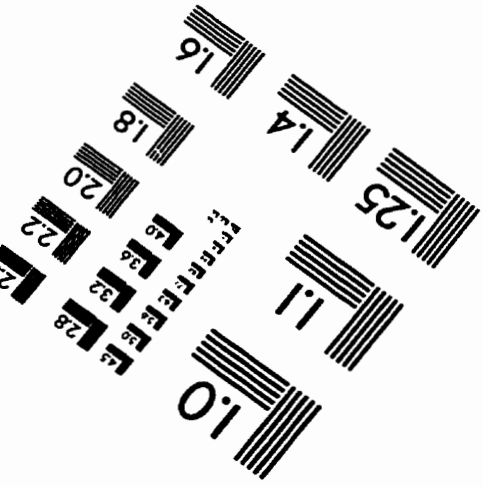

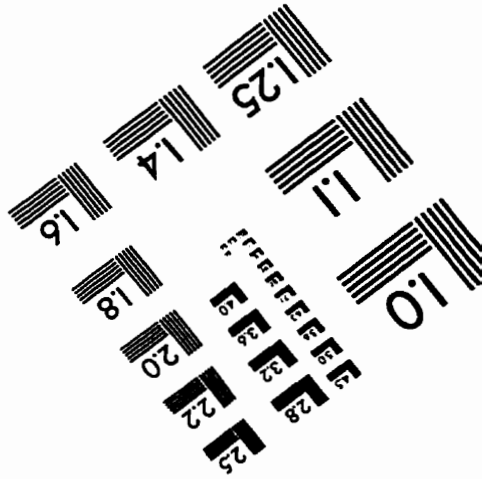

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