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Immunogold localization of mitochondrial aspartate aminotransferase in mitochondria and on the cell surface in normal rat tissues

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Summary. Mitochondrial aspartate aminotransferase (mAspAT) (E.C. 2.6.1.1), an important enzyme in amino acid metabolism, is identical to a fatty acid-binding protein ($FABP_{pm}$) isolated from plasma membranes of several cell types. Employing a monospecific polyclonal antibody to rat mAspAT, we have used immunogold electron microscopy to study the subcellular distribution of mAspAT in various mammalian tissues. Immunogold labeling of rat tissue sections embedded in LR Gold resin showed strong labeling of mitochondria in all tissues examined (viz. liver, pancreas, pituitary, spleen, heart, kidney, submandibular gland). In addition, strong and specific labeling was also observed at a number of non-mitochondrial sites including various locations in kidney, such as on cell surface in distal tubules and cortical collecting ducts, in condensing vacuoles, along cell boundaries between adjoining cells, and in endothelial cells lining capillaries in the glomerulus. Surface labeling due to mAspAT was also seen in arteriolar endothelial cells and in lymphocytes. These findings support the previous identification of mAspAT as both a mitochondrial enzyme and a plasma membrane protein. It is suggested that in accordance with its established role in other cells and tissues, the surface-located mAspAT in kidney and endothelial cells is involved in the fatty acid transport process. The dual-localization of mAspAT, as well as a large number of other mitochondrial proteins (viz. Hsp60, Hsp10, Cytochrome c, TRAP-1 and P32 (gC1q-R)) in recent studies, within both mitochondria and at various specific extramitochondrial sites raises fundamental questions about the role of mitochondria in cell structure and function, and about the mechanisms that exist in normal cells for protein translocation from mitochondria to other compartments. These results have implications for the role of mitochondria in apoptosis and different diseases.

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Introduction

Mitochondrial aspartate aminotransferase (mAspAT) (E.C. 2.6.1.1) is a well studied enzyme that plays a major role in amino acid metabolism and provides an important route for the import of reducing equivalents into mitochondria through its participation in the malate:aspartate shuttle (Christen and Metzler, 1985). In their studies initially unrelated to mAspAT, Berk et al. previously identified a 43 kDa plasma membrane fatty acid-binding protein ($FABP_{pm}$) in a number of different tissues, which was responsible for the facilitated uptake of long chain free fatty acid (FAA) in cells (Stremmel et al., 1985, 1986; Schwieterman et al., 1988). Structural analysis of highly purified $FABP_{pm}$ from rat liver plasma membrane, led to the unexpected discovery that $FABP_{pm}$ was very similar to mAspAT (Berk et al., 1990). Subsequent studies demonstrated that the two proteins were both immunologically and functionally identical (Stump et al., 1993). For example, *in vivo* transfection of NIH 3T3 cells with mAspAT cDNA led to *de novo* expression on the cell surface of a 43 kDa protein immunoreactive with antibodies to both mAspAT and $FABP_{pm}$ (Isola et al., 1995). Moreover, after transfection, these cells, normally deficient in FFA uptake, exhibited saturable uptake of FFA that was selectively inhibitable by antibodies to mAspAT (Isola et al., 1995). Further evidence of a role for mAspAT in FFA uptake derives from studies in HuH7 hepatoblastoma cells. Culture of these cells in ethanol induced a significant increase in the expression of mAspAT mRNA, which was highly correlated with an increase both in the amount of mAspAT protein on the cell surface and increased facilitated uptake of FFA (Zhou et al., 1998). Thus, mAspAT appears to be a member of a growing group of mitochondrial proteins that have different functions in

different cellular compartments (Smalheiser, 1996; Jeffery, 1999; Soltys and Gupta, 1999, 2000).

In the past, work on the sub-cellular distribution of mAspAT has focused almost exclusively on the liver, and the extent to which mAspAT is present at extramitochondrial locations in other tissues is not known. Since mAspAT is a highly conserved protein, with extensive immunological cross-reactivity among mammalian species (Christen and Metzler, 1985; Winefield et al., 1995), in the present report, we have used a highly specific polyclonal antibody to rat liver mAspAT to examine the subcellular distribution of mAspAT in various rat tissues using immunoelectron microscopy. Our results confirm the inference that mAspAT is primarily a mitochondrial protein that is abundantly present in mitochondria of all cells and tissues examined. However, our studies also provide strong evidence that significant quantities of mAspAT are also present on the plasma membrane of several, but not all, of the tissues examined. These results support the concept that while mAspAT is primarily a mitochondrial protein, it is also present and functions at extramitochondrial sites, as has recently been reported for several other mitochondrial proteins (Soltys and Gupta, 1996, 1999, 2000; Cechetto et al., 2000; Cechetto and Gupta, 2000; Soltys et al., 2000; 2001; Sadacharan et al.,, 2001).

Materials and methods

Antibodies

Rabbit polyclonal antibodies to purified rat liver mAspAT were raised and characterized as described previously (Stremmel et al., 1986; Stump et al., 1993; Isola et al., 1995). The sources of other antibodies that were used in this work are as follows: goat anti-rabbit IgG-20nM gold conjugate (British Bio-Cell); anti-human amylase rabbit polyclonal antibody (Sigma, St Louis, MO); anti-bovine catalase sheep polyclonal antibody (Serotec; U.K.); and anti-sheep growth hormone rabbit polyclonal antibody (ICN; Montreal, Quebec, Canada).

Immunoelectron microscopy

Preparation and antibody labeling of rat tissues was performed as previously described (Soltys and Gupta, 1996; Cechetto et al., 2000; Cechetto and Gupta, 2000; Soltys et al., 2000), with some modifications. Sections from liver, pancreas, kidney, pituitary, submandibular glands, spleen and heart were preabsorbed at room temperature with 20% fetal calf serum in 0.1M Tris-HCl, pH 7.5 (carrier buffer). Sections were then reacted with polyclonal antibody to mAspAT (1:25 dilution) in carrier buffer for 2 hours at 37 °C in a humidified incubator. Sections were washed three times with 1% bovine serum albumin (BSA) in 0.1M Tris-HCl, pH 7.5 over a period of 15 min. For secondary labeling, sections were reacted with a 1:10 dilution of goat anti-rabbit IgG-20nm gold conjugate in carrier buffer for 4 hours at 37 °C in a humidified incubator. Sections were then given a high salt wash with 0.5M KCl in carrier buffer, followed by a final wash in H_2O. The sections were then stained with 4% uranyl acetate in 25% ethanol for 15 minutes and then washed with 25% ethanol. Sections were examined at 80 kV with a JEOL 1200 EX transmission electron microscope.

Results

Subcellular localization of mAspAT in normal rat tissues

The antibody to mAspAT used in the present work has been characterized extensively. In Western blots of tissue extracts, plasma membrane preparations, as well as in purified rat liver mitochondrial fractions (unpublished results), it reacts with a single band of 43-kD, corresponding to mAspAT. Immunofluorescence studies with this antibody in permeabilized mouse 3T3 cells show specific labeling of mitochondria. However, in non-permeabilized 3T3 cells, particularly those which overexpress the cDNA for mAspAT, strong and specific labeling on cell surface was also observed. Subcellular localization of mAspAT in rat tissue sections, prepared as described in Materials and Methods, was carried out using immunogold electron microscopy. Tissue sections embedded in LR Gold were probed with polyclonal antibodies to mAspAT, followed by detection with secondary antibodies conjugated to 20 nm gold particles. The specificity of the secondary antibodies used in this study was confirmed by carrying out the labeling of tissue sections without the primary antibody, which resulted in abolishment of all labeling in different tissues (results not shown). This provides evidence that the secondary antibodies bind specifically to the primary antibody and do not react with the acrylic resin in which the tissue is embedded. The results of our studies with different tissues are as follows:

Liver

Subcellular localization of mAspAT in a representative rat liver LR Gold tissue section is shown in Figure 1. As seen, the antibody showed strong labeling of mitochondria, and the labeling in other compartments, such as nucleus, ER or peroxisomes was either completely absent or was at a very low background level. The lack of labeling in peroxisomes is of interest because in our earlier work, antibodies to another mitochondrial protein, Hsp60, have been shown to label these structures. The identity of peroxisomes was based on their specific labeling with antibody to catalase (not shown).

Pancreas

Figures 2A,B show the results of labeling in pancreatic acinar cells and insulin secretory cells,

respectively. In pancreatic acinar tissue sections (Fig. 2A), the antibody to mAspAT showed strong labeling of mitochondria. In comparison, the labeling in other compartments, such as nucleus, ER and zymogen granules (ZG) was very low and close to background levels. The weak, or lack of, reactivity of mAspAT antibody with zymogen granules is of particular interest because antibodies to a variety of other mitochondrial proteins that we have previously examined (viz. Hsp60, TRAP-1, P32 (gC1q-R) protein and cytochrome c), all showed significant reactivity in these structures. In pancreatic insulin secretory cells labeling with mAspAT antibody was seen mainly in mitochondria (Fig. 2B). Labeling was not seen in insulin secretory granules and the labeling in cytoplasm and nucleus was near background levels.

Pituitary and submandibular glands

Mitochondrial labeling was also seen in anterior pituitary (Fig. 3) and serous cells found in the submandibular gland (not shown). In the anterior pituitary labeling was specific for mitochondria. The growth hormone granules where antibodies to a number of other mitochondrial proteins have previously been shown to localize were not labeled. Likewise, in serous cells found in the submandibular gland, labeling was again specific for mitochondria with hardly any labeling seen in secretory granules (not shown). The labeling in the cytoplasm and nucleus in both these tissues was very low and close to background levels.

Kidney

In contrast to the tissues described thus far, mAspAT localized to a variety of extramitochondrial locations in different regions of the rat kidney. In the distal convoluted tubules, mAspAT reactivity was found in mitochondria and also in dense structures (marked V) which appear to correspond to condensing vacuoles (Fig. 4A). No significant labeling was seen in any other compartments. In the ascending part of the thick distal tubule of rat kidney, both specific mitochondrial labeling and labeling of the basal region of the cell surface were observed (Fig. 4B). The labeling on the basal cell surface is not homogenous, and seems to be concentrated in regions with protrusions.

Surface labeling for mAspAT was also seen in other regions of the kidney, such as cell boundaries of adjoining kidney cells (Fig. 5A). The identity of cells showing such labeling is presently unclear. Specific labeling was also observed in mitochondria, and no

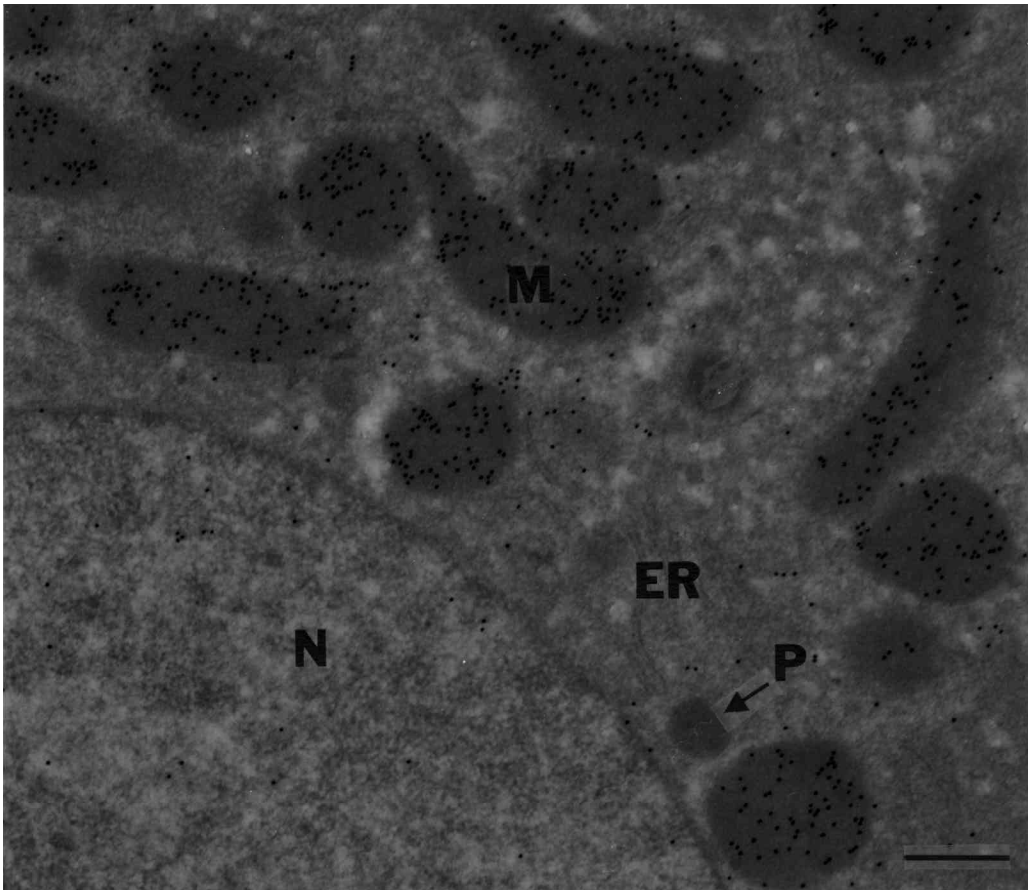
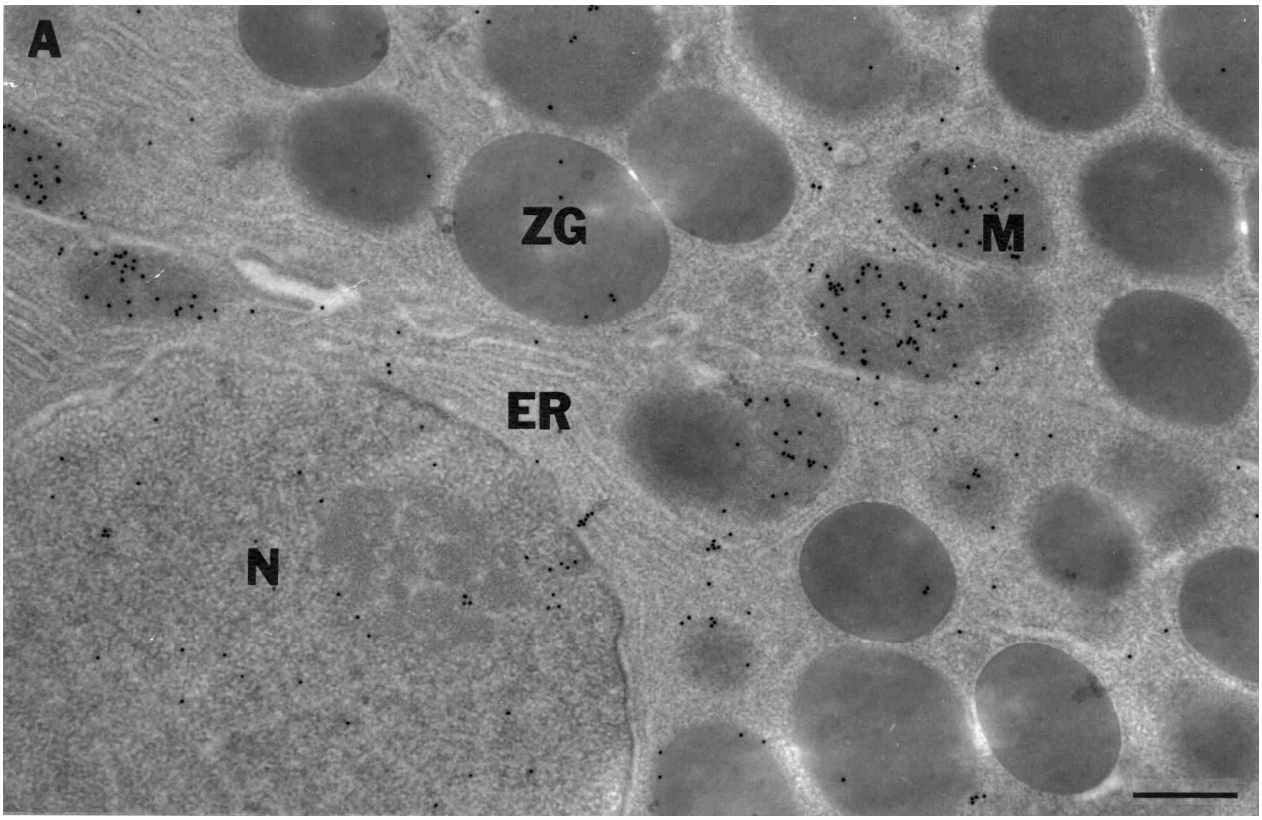


Fig. 1. Subcellular localization of mAspAT in rat liver LR Gold sections using immunogold electron microscopy. Labeling is carried out using a polyclonal antibody to mAspAT and then followed by secondary antibody bound to 20-nm gold particles. Labeling is seen mainly in mitochondria with no labeling present in peroxisomes. N: nucleus; M: mitochondria; P: peroxisomes; ER: endoplasmic reticulum. Bar: 500 nm.



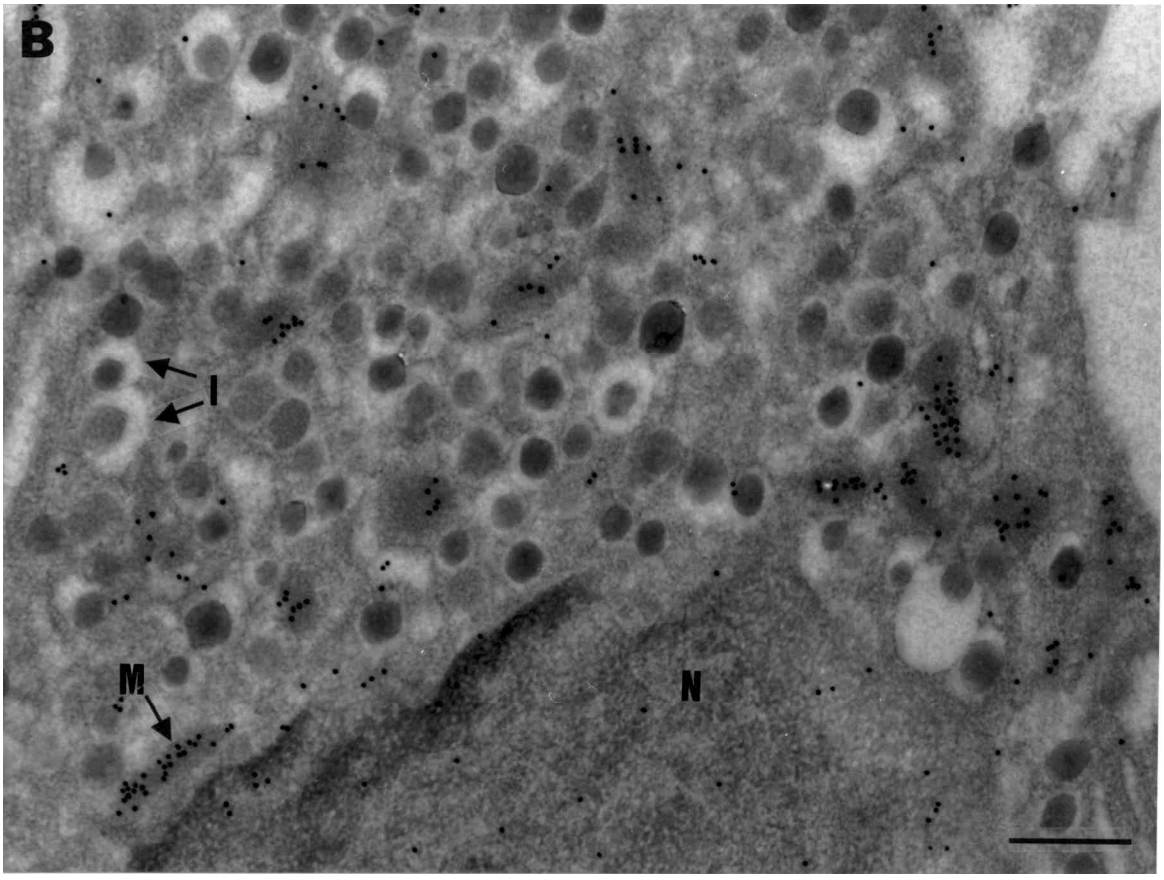


Fig. 2. Subcellular localization of mAspAT in rat pancreas, using immunogold electron microscopy. **A.** Reactivity in pancreatic acinar cell LR Gold sections is seen mainly in mitochondria; labeling seen in other compartments is close to background levels. **B.** Localization of mAspAT within mitochondria in insulin secretory cells. No reactivity is seen in the insulin secretory granules. 20-nm gold particles. N: nucleus; M: mitochondria; ER: endoplasmic reticulum; ZG: zymogen granules; I: insulin secretory granule. Bar: 500 nm.

significant labeling was seen in the cytosol or nucleus of these cells. Figure 5B, shows the specific labeling of mAspAT along the luminal side of endothelial cells lining capillaries of rat kidney glomerulus. Although no blood cells were present within the capillaries they were identified as capillaries based on the orientation of the pedicles. Reactivity for mAspAT in kidney cortical collecting duct tissue sections was found exclusively in mitochondria and on the apical cell surface (Fig. 6). It is to be noted that the labeling seen on cell surface is not uniform but is generally restricted to cell surface protrusions along the microvilli. In contrast to the distal convoluted region, in the proximal convoluted tubule region of rat kidney, labeling was seen only in mitochondria with no surface labeling observed in the brush border region (not shown).

Other sites

Surface localization of mAspAT was also seen in endothelial cells lining arterioles (Fig. 7A). The labeling seen on the surface in this case was again not uniform, but concentrated in the projections present on the cell surface. It is of note that similar labeling has also been observed for TRAP-1 and P32 proteins, both of which are mitochondrial proteins. mAspAT reactivity was also seen on the cell surface in lymphocytes (Fig. 7B). The lymphocyte surface labeling was also not uniform and resembled that on the endothelial cell surface. Significant labeling was not seen at any other location in lymphocytes. In a number of other tissues studied, namely spleen and heart, prominent labeling was again seen for mitochondria and no significant labeling was observed at any other location (not shown).

Discussion

mAspAT has previously been identified as both a mitochondrial enzyme and, in a limited number of cell types, as a plasma membrane fatty acid-binding protein. Although the presence of mAspAT on plasma membranes has been investigated previously by immunofluorescence and Western blotting in several tissues and in cell lines (Stremmel et al., 1985, 1986; Berk et al., 1990; Stump et al., 1993; Isola et al., 1995; Zhou et al., 1998), and by immunoelectron microscopy in a rat hepatoblastoma cell line (Zhou et al., 1998), the current study represents the first detailed application of immunoelectron microscopic methods to examine the subcellular localization of mAspAT in multiple tissues. Our survey of different tissues for mAspAT localization shows that although mAspAT is abundantly found in mitochondria in all cases, significant and specific reactivity to mAspAT antibody was also found at the cell

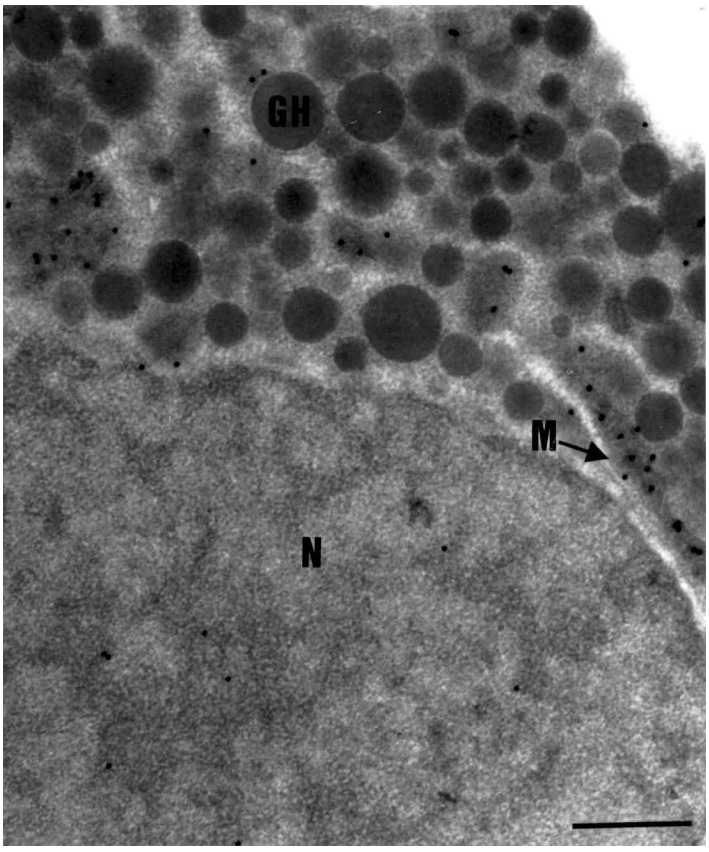


Fig. 3. Mitochondrial localization of mAspAT in rat anterior pituitary 20-nm gold particles. N: nucleus; M: mitochondria; GH: growth hormone granules. Bar: 500 nm.

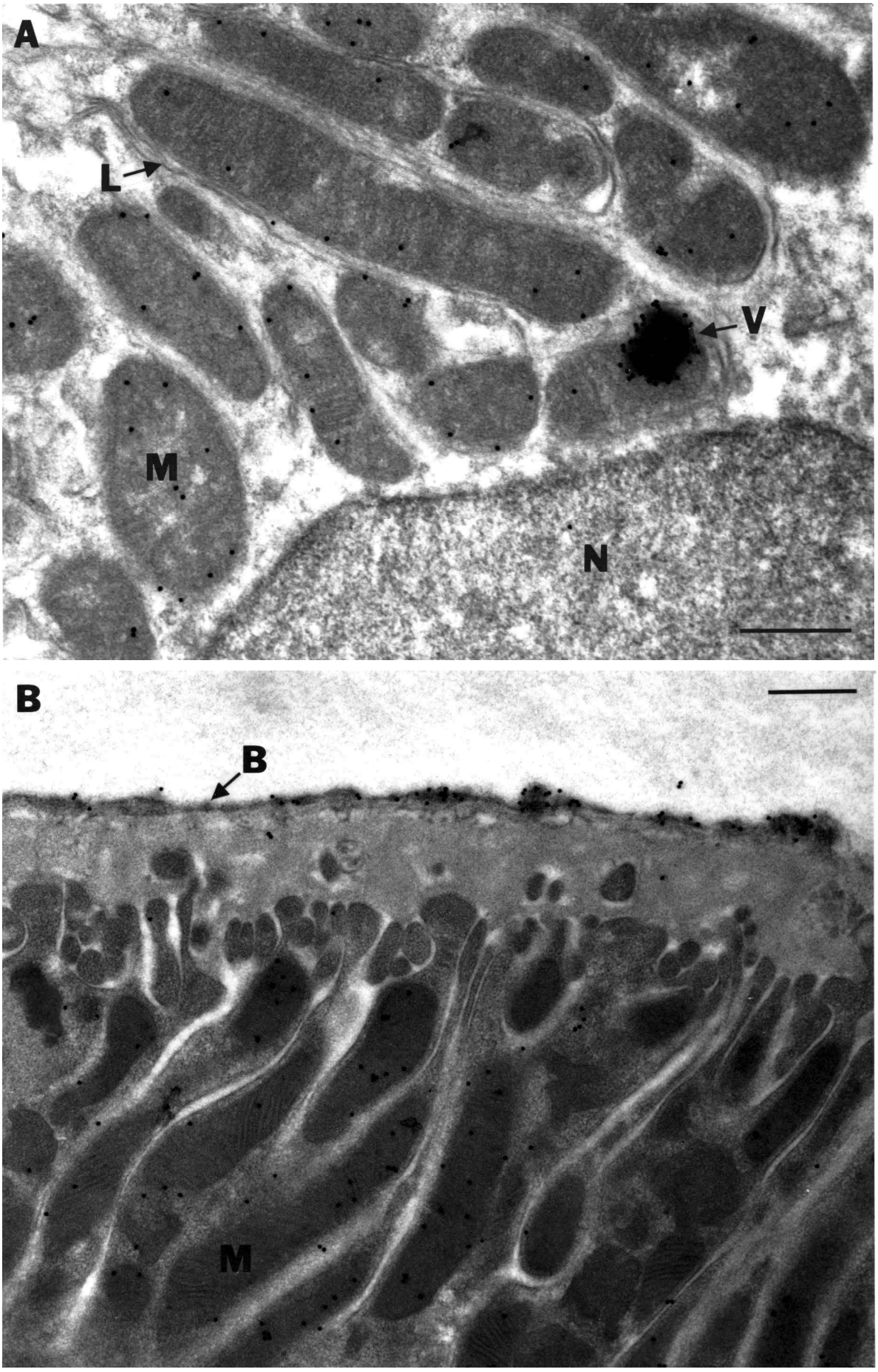


Fig. 4. Extra-mitochondrial reactivity with mAspAT antibody in distal tubule region from rat kidney. **A.** In the distal convoluted tubule of rat kidney, labeling is seen in condensing vacuoles as well as in mitochondria. **B.** In the ascending part of thick distal tubule of rat kidney, labeling is seen on the basal plasma membrane in addition to mitochondria. Secondary antibody used was conjugated to 20-nm gold particles. N: nucleus; M: mitochondria; L: lateral intercellular labyrinth; V: condensing vacuoles; B: basal membrane. Bars: 500 nm.

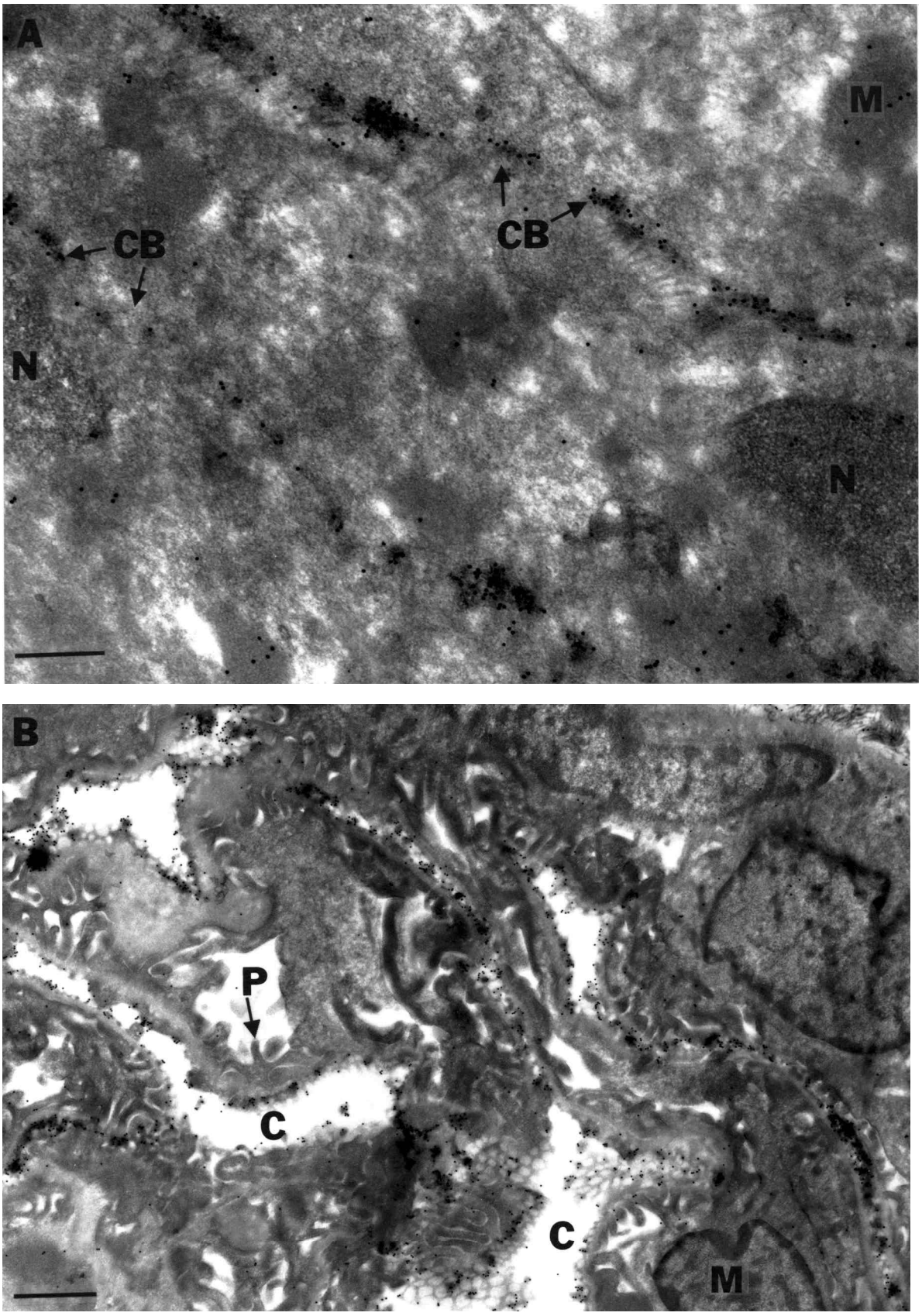


Fig. 5. Cell surface reactivity of mAspAT antibody in rat kidney. **A.** In three adjoining rat kidney cells labeling is seen primarily within mitochondria and along the cell boundaries. N: nucleus; M: mitochondria; CB: cell boundary; P: pedicels; Bar: 500 nm. **B.** In rat kidney glomerulus, labeling is along the luminal side of endothelial cells lining capillaries. C: capillaries; M: mesangial cells. Bar: 1000 nm.

surface in a number of tissues. While the presence of mAspAT in mitochondria was expected in view of extensive earlier work identifying this organelle as the principal site of its enzymatic functions (Christen and Metzler, 1985), the localization of mAspAT on the cell surfaces in various regions of the kidney and in arteriolar endothelial cells represents a novel finding that is of considerable significance. The surface labeling due to mAspAT was not found in all tissues, but restricted to only certain tissues, and even within each tissue reactivity was seen only in certain specialized cells, as exemplified by the findings in rat kidney. This suggests that mAspAT is specifically targeted to the cell surface only in certain cells or tissues to meet specific functional needs. These observations raise two obvious questions: (i) What is the trafficking pathway by which mAspAT reaches the plasma membrane? and (ii) what is the functional significance of its presence there?

With regard to the first of these questions, it is well established that mAspAT is encoded by nuclear DNA, and is translated on free cytosolic ribosomes as a larger pre-protein containing an N-terminal mitochondrial targeting sequence, which is not present in the mature protein (Sonderegger et al., 1982). Since the targeting sequence is encoded by exon 1 of the mAspAT gene, with the exon 1:exon 2 splice site occurring at the first codon of the mature protein (Joh et al., 1985; Lain et al., 1998), it was originally considered that the distribution of the protein to extramitochondrial sites might be the consequence of alternative RNA splicing, creating several mAspAT mRNAs having different targeting sequences. However, subsequent studies have established that there is only a single message for mAspAT in cells in which there is abundant mAspAT on the plasma membrane, indicating that the distribution of mAspAT protein to different sub-cellular sites does not reflect alternative RNA splicing and creation of multiple messages (Bradbury and Berk, 2000). mAspAT isolated from plasma membranes of hepatocytes, adipocytes, and cardiac myocytes has been shown to be the mature form of the protein lacking the N-terminal targeting presequence (Stremmel et al., 1985; Schwieterman et al., 1988; Berk et al., 1990; Stump et al., 1993). In our earlier work with Hsp60 protein, which is also found at a variety of extramitochondrial sites, evidence has been presented that the conversion of the precursor form of the protein to its mature form found at other sites, does not occur if the mitochondrial import of the preprotein is inhibited (Soltys and Gupta, 1996). These results favor the possibility that the proteins are first imported into mitochondria and, after cleavage of the pre-sequence, they are translocated to the other sites (Soltys and Gupta, 1999, 2000). The mechanism(s) by which proteins are translocated from mitochondria to other sites is presently unclear, although a number of possibilities have been discussed (Soltys and Gupta, 2000).

There is now extensive evidence supporting the view that the mAspAT on the plasma membrane is central to

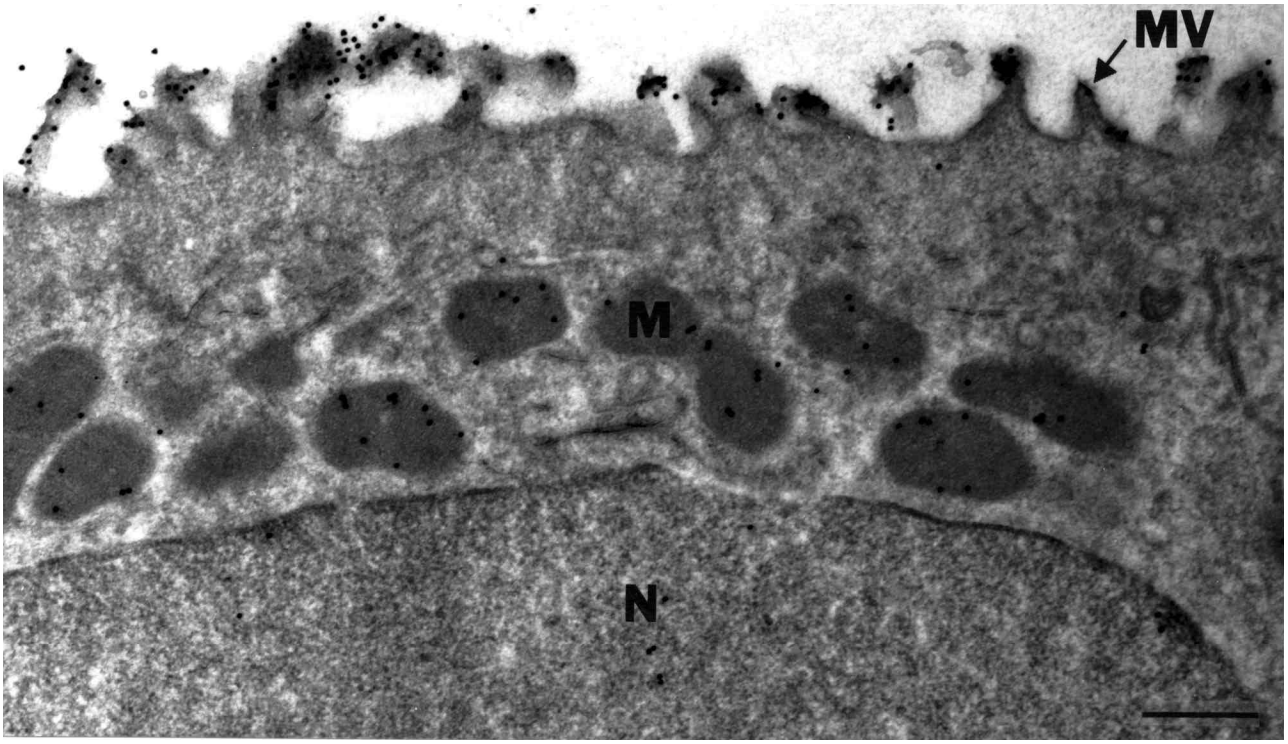


Fig. 6. Subcellular localization of mAspAT in rat kidney cortical collecting duct region. Reactivity for mAspAT is seen mostly in mitochondria and surface microvilli. N: nucleus; M: mitochondria; MV: microvilli. Bar: 500 nm.

the process of facilitated FFA uptake and efflux (Stremmel et al., 1985, 1986; Schwieterman et al., 1988; Berk et al., 1990; Stump et al., 1993; Isola et al., 1995; Zhou et al., 1998; Berk and Stump, 1999), in many different cells and tissues, of which isolated hepatocytes and hepatoblastoma cell lines have been most extensively studied. In this context, our observation that the antibodies to mAspAT show strong and specific labeling of cell surface in various regions of the kidney, is of much interest. The different regions of kidney where mAspAT is specifically localized include the basal cell surface in distal convoluted tubule region, in

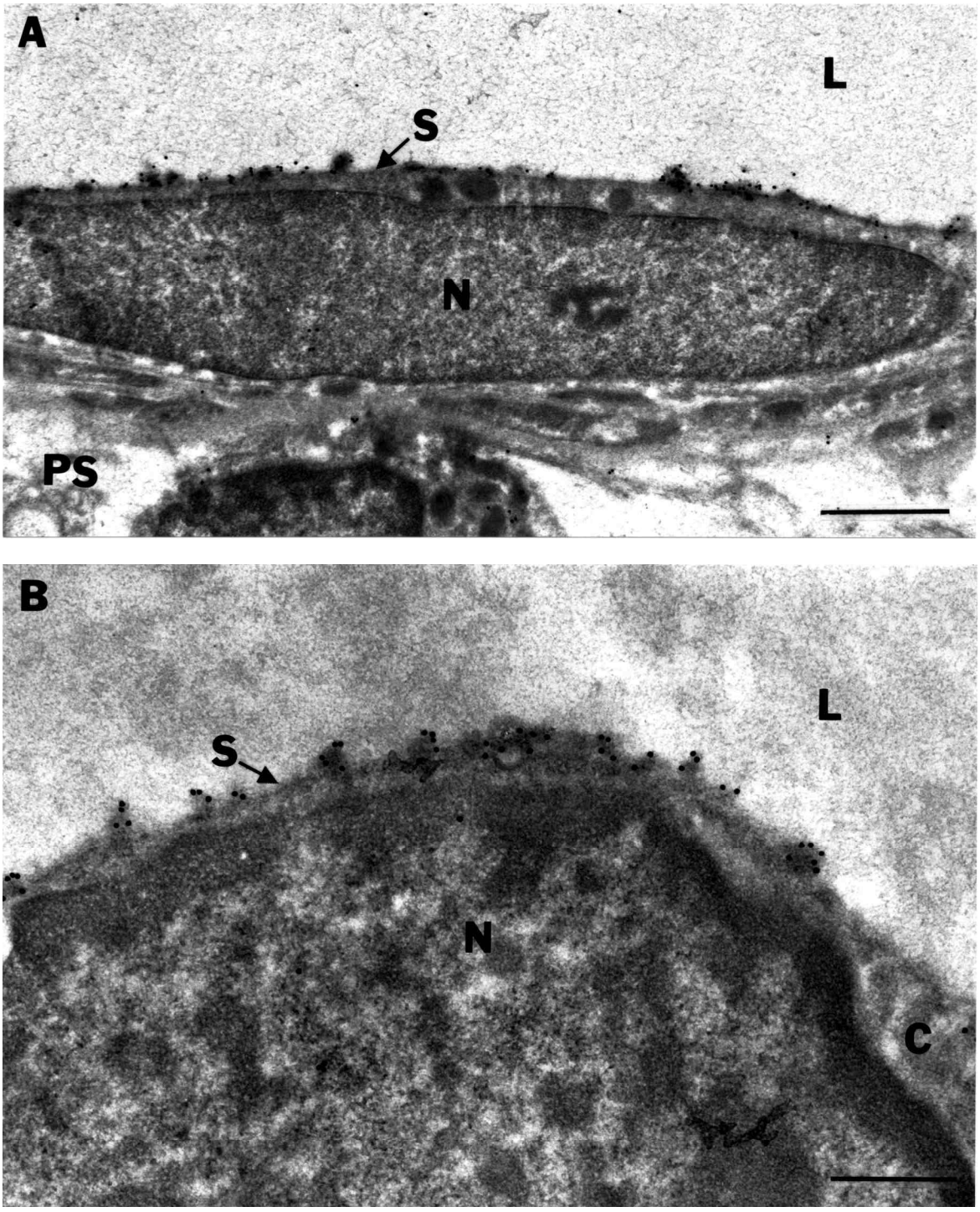


Fig. 7. Surface labeling due to mAspAT antibody in cells from the circulatory system.
A. Surface labeling of mAspAT is seen in an endothelial cell lining an arteriole in rat kidney.
Bar: 1000 nm.
B. Surface reactivity is seen in a lymphocyte present in a blood vessel from rat kidney.
N: nucleus;
L: lumen;
S: surface;
PS: pericapillary space;
C: cytosol.
Secondary antibody used conjugated to 20 nm gold particles.
Bar: 500 nm.

condensing vacuoles which are a part of the endocytotic apparatus on the apical cell surface (Bulger, 1988), microvilli of cortical collecting duct cells, and along the cell boundaries of adjoining cells in an unidentified region of the kidney. In contrast, no mAspAT reactivity was observed at either the apical or basal cell surface of the proximal convoluted tubule. Although the renal uptake or disposition of FFA has not been extensively studied, in part because these tightly albumin-bound compounds normally experience little or no glomerular filtration, our observation that mAspAT is present in appreciable amounts in these specific kidney regions, provides strong suggestive evidence that these regions of kidney are actively engaged in the uptake and/or efflux of FFA.

In addition to the different regions in kidney, specific cell surface labeling was also observed in endothelial cells lining blood vessels and in lymphocytes. The surface labeling in these cells, as also in the case of kidney, was not uniform but localized to specific regions, often in the form of membrane projections which could comprise of vesicles that were either fusing with or budding from the membrane. The need for specific transport mechanisms in endothelial cells to move FFA across the endothelial barrier from the vascular lumen to the extravascular, extracellular space has been speculated upon (Vyska et al., 1991; Berk and Stump, 1999) and our results provide suggestive evidence for the existence of such a process in these cells. Thus, the various sites on the plasma membrane where mAspAT is seen may have in common the shared characteristics of facilitated FFA transport processes. Conversely, neither the existence of nor an apparent need for facilitated FFA transport is evident in acinar or insulin secretory cells of the pancreas, the anterior pituitary or submandibular gland, sites at which no mAspAT was detected on the plasma membrane.

Our observation that mAspAT, which is primarily a mitochondrial protein, is also found on cell surface in many different cells and tissues, where it is apparently involved in FFA transport, raises fundamental questions concerning the cellular functions of mitochondrial proteins. In recent years, many other examples have been reported of proteins that, like $FABP_{pm}$, were initially identified on the basis of non-mitochondrial functions, but were subsequently identified as mitochondrial proteins (Soltys and Gupta, 1999, 2000). These proteins include the mitochondrial heat shock protein Hsp60, which has been implicated in resistance to antimitotic drugs (Picketts et al., 1989; Gupta, 1990), amino acid transport (Jones et al., 1994), signal transduction (Ikawa and Weinberg, 1992; Khan et al., 1998), and immune response (Kaur et al., 1993; Khan et al., 1998); the mitochondrial heat shock protein Hsp70, which has been implicated in cellular senescence (Wadhwa et al., 1993) and antigen presentation (Domanico et al., 1993); the Hsp60 co-chaperone Hsp10, which has been shown to be identical to the early pregnancy growth factor found in maternal blood (Cavanagh, 1996; Sadacharan et al., 2001); the tumor necrosis factor receptor associated protein TRAP-1 (Cechetto and Gupta, 2000; Felts et al., 2000), which has been shown to interact with the type I tumor necrosis factor and the retinoblastoma protein (Chen et al., 1996); the ß-subunit of mitochondrial F1-ATPase, reported to occur on the basolateral domain of the hepatocyte plasma membrane and to function as an organic anion binding protein (Wolkoff and Chung, 1980; Goeser et al., 1990); the protein p32, involved in nuclear functions as well as acting at the cell surface as a receptor for complement C1q (Muta et al., 1997; Soltys et al., 2000); and a growing number of others. The work on these and other proteins has been recently reviewed by us (Soltys and Gupta, 1999, 2000). Most interestingly, our recent work shows that cytochrome c, whose release from mitochondria is believed to play a central role in the cascade of events leading to apoptotic cell death (Green and Reed, 1998; Desagher and Martinnou, 2000), is also present outside mitochondria in a variety of compartments including pancreatic zymogen granules and condensing vacuoles as well as in growth hormone granules, in normal tissues (Soltys et al., 2001). These results indicate that the translocation of cytochrome c from mitochondria to other specific sites also occurs under normal physiological conditions and its release in cytoplasm leading to apoptosis could be due to perturbation of the normal pathways.

It is currently believed that the primary function of mitochondria and mitochondrial resident proteins is related to oxidative phoshporylation and that under normal physiological conditions these proteins do not exit mitochondria or play any functional roles outside of mitochondria (Scheffler, 1999). In this context, our observation that a large number of well-characterized mitochondrial proteins, including mAspAT, which were previously thought to reside and function exclusively in mitochondria, are present at a variety of specific locations in cells and involved in different functions have led us to postulate the existence of specific mechanism(s) by which certain proteins could exit mitochondria to assume additional functions at specific extramitochondrial sites (Soltys and Gupta, 1999, 2000). Although such mechanisms remain to be characterized, these observations strongly indicate that the cellular functions of mitochondrial proteins are not restricted to within mitochondria and that mitochondria and mitochondrial-resident proteins play an important role in diverse cellular processes (Soltys and Gupta, 1999, 2000). In recent years it has become clear that mutations affecting mitochondrial components are responsible for a broad spectrum of genetic diseases affecting many different tissues and organs, often in a highly specific manner (Wallace, 1999; Schon, 2000). Based on the known function of mitochondria in oxidative phosphorylation, it has proven difficult to logically explain these observations (Schon, 2000). However, our observations that mitochondrial proteins are involved in a variety of extraneous functions provide insight that could prove useful in understanding the role of

mitochondria in the etiology of different diseases.

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