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Methionine Aminopeptidase II: A Molecular Chaperone for Sarcoplasmic Reticulum Calcium ATPase

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Abstract The monoclonal antibody to the β-subunit of H^+/K^+-ATPase (mAbHKβ) cross-reacts with a protein that acts as a molecular chaperone for the structural maturation of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase. We partially purified a mAbHKβ-reactive 65-kDa protein from *Xenopus* ovary. After in-gel digestion and peptide sequencing, the 65-kDa protein was identified as methionine aminopeptidase II (MetAP2). The effects of MetAP2 on SR Ca^{2+}-ATPase expression were examined by injecting the cRNA for MetAP2 into *Xenopus* oocytes. Immunoprecipitation and pulse-chase experiments showed that MetAP2 was transiently associated with the nascent SR Ca^{2+}-ATPase. Synthesis of functional SR Ca^{2+}-ATPase was facilitated by MetAP2 and prevented by injecting an antibody specific for MetAP2. These results suggest that MetAP2 acts as a molecular chaperone for SR Ca^{2+}-ATPase synthesis.

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Introduction

Among typical cation-transporting P2-type ATPases, the plasma membrane Na^+/K^+-ATPase and the gastric H^+/K^+-ATPases consist of a catalytic α- and a glycosylated β-subunit, whereas the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is composed of only an α-subunit. Almost all the functions of these three ATPases have been attributed to the highly conserved α-subunit (Hilge et al. 2003; Kuhlbrandt 2004). The β-subunit is absolutely required for correct folding of the Na^+/K^+- and H^+/K^+-ATPase α-subunits in the membrane (Noguchi et al. 1987, 1990; Colonna et al. 1997; Beguin et al. 1998; Geering 2001). The question we address in this work is how the SR Ca^{2+}-ATPase may fold correctly in the membrane without the help of a β-subunit.

Previously, we demonstrated that the β-subunits of Na^+/K^+- and H^+/K^+-ATPase transiently associated with, and thereby assisted in, the correct positioning of SR Ca^{2+}-ATPase in *Xenopus* oocytes (Noguchi et al. 2003). SR Ca^{2+}-ATPase synthesis in oocytes was prevented by injection of an anti-β-subunit antibody. These observations suggested the existence of an endogenous β-subunit-like protein in oocytes that assisted in SR Ca^{2+}-ATPase synthesis, such as a molecular chaperone.

Here, using an anti-β-subunit antibody to probe *Xenopus* oocytes, we identified methionine aminopeptidase 2 (MetAP2) as an endogenous molecular chaperone for SR Ca^{2+}-ATPase.

Materials and Methods

Materials

The plasmids used for cRNA synthesis of Na^+/K^+-ATPase and SR Ca^{2+}-ATPase were previously described (Noguchi et al. 1987; Kawamura and Noguchi 1991). The cDNA encoding *Xenopus* MetAP2 was prepared from *Xenopus* poly-A RNA using a Fast Track 2.0 Kit (Invitrogen, Carlsbad, CA). Reverse-transcriptase polymerase chain reaction was performed with the RNA LA PCR Kit (TaKaRa, Shiga, Japan) using a primer (5′-GCGGGCTTAGTGGTAA AGC-3′) designed from the *Xenopus* MetAP2 sequence (BC043889-1) and an oligo dT-adaptor primer (TaKaRa) according to the manufacturer's instructions. The 1.9-kb product was cloned into pGEM-T (Promega, Madison, WI), and clone pGEMMAP2 was isolated, where sequencing confirmed the MetAP2 cDNA in the same orientation as the SP6 promoter. pGEMMAP2 was linearized and used as the template for cRNA synthesis (MEGAscript SP6 Kit; Ambion, Austin, TX). The antiserum to rabbit SR Ca^{2+}-ATPase was a gift from H. Suzuki (Asahikawa Medical College, Asahikawa, Japan). The antiserum to the β-subunit of *Torpedo* Na^+/K^+-ATPase was raised in rabbits as previously described (Noguchi et al. 1987). The polyclonal antibody against human MetAP2 and the monoclonal antibody against pig H^+/K^+-ATPase β-subunit (mAbHKβ) were purchased from Zymed Laboratories (South San Francisco, CA) and Affinity BioReagents (Golden, CO), respectively.

cRNA Expression in *Xenopus* Oocytes

Xenopus laevis oocytes (stage V-VI) were injected with cRNAs encoding for rabbit SR Ca^{2+}-ATPase, *Xenopus* MetAP2 or the β-subunit of *Torpedo* Na^+/K^+-ATPase, separately or in combination (10 ng of each cRNA/23 nl water for each oocyte). The oocytes were incubated at 19°C for 8-12 h in modified Barth's medium (MBM) containing 100 μg/ml each of ampicillin and streptomycin, followed by labeling with [^{35}S]-methionine and [^{35}S]-cysteine (EXPRE^{35}S^{35} protein labeling mix, 1,175 Ci/mmol; NEN, Boston, MA). At appropriate time intervals, the oocytes were harvested and the samples quickly frozen in dry ice-ethanol. Immunoprecipitation was carried out as previously described (Noguchi et al. 1994), and autoradiographic images were produced and quantified on a FLA2000 Bioimage Analyzer (FJIX, Tokyo, Japan). All experiments were performed at least twice, and the results were reproducible.

Pulse-Chase Analysis

After 8–12 h of incubation in MBM, the cRNA-injected oocytes were radiolabeled by incubating in radioactive amino acid-containing medium (EXPRE^{35}S^{35}, 0.3 mCi/ml) at 19°C for 1 h. The labeled oocytes were transferred to the chase medium containing unlabeled methionine and cysteine at 100-fold excess over the radiolabeled amino acids and incubated at 19°C for various time intervals.

Ca^{2+}-ATPase Activity Assay

About 150 oocytes were homogenized in 1.5 ml of 50 mM Tris/HCl buffer (pH 7.5) containing 0.25 M sucrose and 1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride hydrochloride (APMSF). The homogenate was centrifuged in a swinging bucket rotor at 7,000 × *g* for 10 min on a 50% sucrose cushion (0.3 ml) to remove the yolk granule, and the resulting supernatant was further centrifuged at 160,000 × *g* for 30 min. After treatment with 0.475 M NaSCN as previously described (Noguchi et al. 1990), the microsomal pellets were resuspended in 0.25 M sucrose and 50 mM Tris/HCl buffer (pH 7.5), and 10–20 μg microsomes were assayed for Ca^{2+}-ATPase activity colorimetrically using the malachite green method (Lanzetta et al. 1979). The reaction mixture (20 μl) consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2), 0.1 M KCl, 3 mM $MgCl_2$, 5 mM NaN_3, 0.1 mM ouabain, 10 μM $CaCl_2$ and 0.6 mM adenosine triphosphate (ATP) in either the presence or the absence of 3 μM thapsigargin at 37°C for 6 min. The reaction was started by addition of ATP.

Partial Purification and Peptide Sequencing of the 65-kDa Molecular Chaperone-like Protein from Xenopus Ovary

As mentioned in our previous report (Noguchi et al. 2003), the antibodies specific for the β-subunit of the Na^+/K^+- and the H^+/K^+-ATPases cross-reacted with a chaperone-like protein for the SR Ca^{2+}-ATPase in *Xenopus* oocytes. Of the anti-β-subunit antibodies we examined, mAbHKβ was chosen as the probe for this work.

Xenopus ovaries were homogenized in a Polytron homogenizer (KINEMATICA AG, Lucerne, Swiss) in 50 mM imidazole/HCl buffer (pH 6.8) containing 0.25 M sucrose, 100 mM KCl and 1 mM APMSF. The homogenate was fractionated by centrifugation into soluble and microsomal fractions. The mAbHKβ-reactive protein was abundant in the soluble fraction rather than the microsomal fraction, so we further purified the molecular chaperone-like protein from the soluble fraction.

An ammonium sulfate fraction between 50% and 60% saturation was collected and subjected to diethylaminoethyl (DEAE) chromatography (DEAE Toyopearl, Tosho, Tokyo, Japan). After Western blot analysis, the DEAE fractions rich in mAbHKβ-reactive protein (65 kDa) were applied to a Q Sepharose Fast Flow (GE Healthcare,

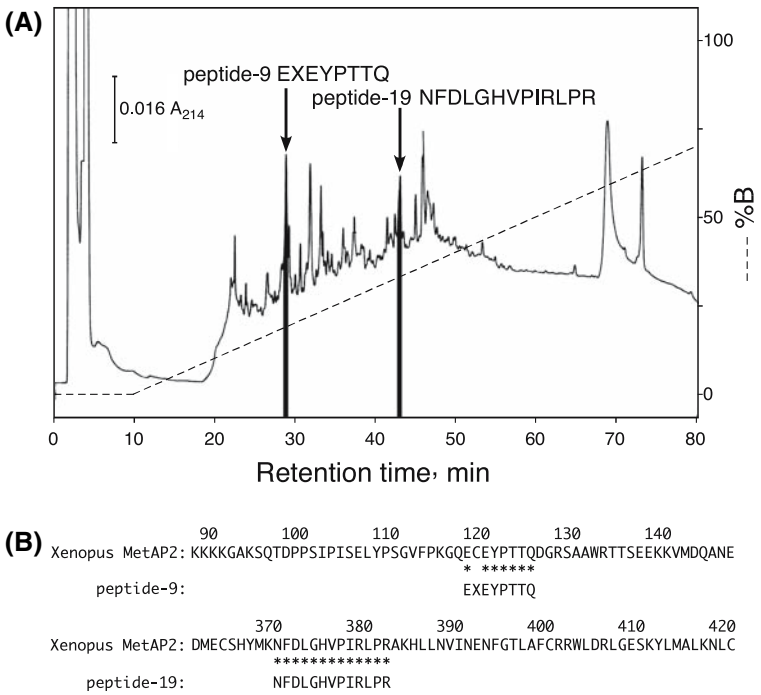

Fig. 1 Peptide sequence of the 65-kDa protein. (*A*) After in-gel digestion, peptides were purified on a C_{18} column (Cosmosil 5C18, ϕ4.6 × 150 mm; Nacalai Tesque) reverse-phase HPLC (solvent A, 0.1% TFA; solvent B, 0.1% TFA and 95% CH_3CN). A_{214} and % B (*dotted line*) are shown. Several peaks were sequenced, and the sequences of two peptides (peptide-9 and peptide-19) are displayed. (*B*) After homology search analysis, the sequences of peptide-9 and peptide-19 were identified in the sequence of *Xenopus* MetAP2 (BC043889-1). They are positioned at residues 118-125 (peptide-9) and 370-382 (peptide-19) of MetAP2

Backinghamshire, UK) column. The fractions rich in 65 kDa protein were combined and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 65-kDa band was visualized with the Gel-Negative Staining Kit (Nacalai Tesque, Kyoto, Japan). Even the most enriched fraction from the Q Sepharose column still contained a few minor proteins in addition to the mAbHKβ-reactive 65-kDa protein. The 65-kDa band was excised and alkylated with iodoacetamide, and the gel pieces were dehydrated with CH_3CN, rehydrated with 25 mM NH_4HCO_3 containing 0.1 μg/ml trypsin and digested at 37°C overnight. The gel pieces were then extracted with 50% CH_3CN containing 5% trifluoroacetic acid (TFA). The extract was subjected to high-performance liquid chromatography (HPLC) with a C_{18} reverse-phase column, and several peak fractions were sequenced (Applied Biosystems, Foster City, CA; model 491).

Results

Characterization of a Molecular Chaperone–like Protein in *Xenopus* Oocytes

Antibodies against the β-subunit of Na^+/K^+- or H^+/K^+-ATPase prevented the synthesis of SR Ca^{2+}-ATPase in *Xenopus* oocytes, possibly by cross-reacting with a protein acting as a molecular chaperone for SR Ca^{2+}-ATPase (Noguchi et al. 2003). This observation suggested that the antibody against the β-subunit could be a useful tool for searching for a molecular chaperone-like protein in oocytes. Of all the ß-subunit antibodies we screened, we chose mAbHKβ as the probe as this antibody had the highest potency at preventing SR Ca^{2+}-ATPase synthesis in *Xenopus* oocytes. As described in Materials and Methods, the mAbHKβ-reactive 65-kDa protein was partially purified from *Xenopus* ovary and subjected to in-gel digestion with trypsin. Two of the peptides generated by digestion were further isolated by C_{18}-reverse phase chromatography and sequenced. Both sequences were found to be of MetAP2 from *Xenopus*, as shown in Figure 1.

Association of SR Ca^{2+}-ATPase and MetAP2

Xenopus oocytes were injected with SR Ca^{2+}-ATPase cRNA (or cRNA encoding the β-subunit of the Na^+/K^+-ATPase, as a control) with or without MetAP2 cRNA. Figure 2 shows the immunoprecipitations with specific antisera followed by autoradiography. When SR Ca^{2+}-ATPase cRNA was coinjected with MetAP2 cRNA, anti-MetAP2 precipitated both expressed proteins, although SR Ca^{2+}-ATPase was precipitated at a much lower level than when precipitated with anti-SR Ca^{2+}-ATPase (Fig. 2, lane 4). Coprecipitation of SR Ca^{2+}-ATPase was not seen when MetAP2 cRNA was injected alone (*data not shown*). When cRNAs encoding MetAP2 and the β-subunit of Na^+/K^+-ATPase were injected together, coprecipitation of the β-subunit and MetAP2 was not observed (Fig. 2, lane 6). Since the anti-MetAP2 does not cross-react with SR Ca^{2+}-ATPase, these observations suggest that SR Ca^{2+}-ATPase and MetAP2 associate specifically with each other. Comparable or lower levels of SR Ca^{2+}-ATPase were

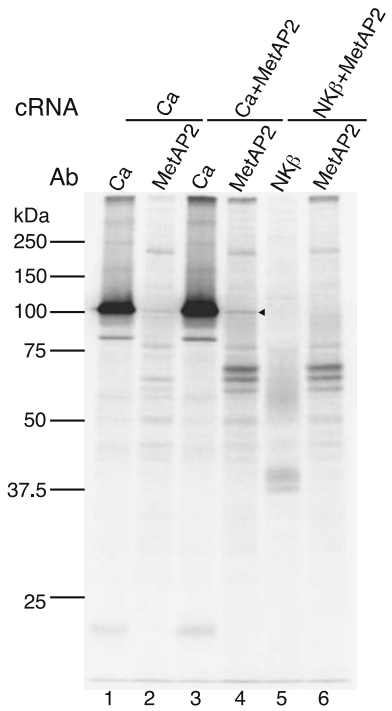

Fig. 2 Coexpression of SR Ca^{2+}-ATPase and MetAP2 in *Xenopus* oocytes. Oocytes were injected with cRNA encoding either the SR Ca^{2+}-ATPase or the β-subunit of Na^+/K^+-ATPase, with or without MetAP2 cRNA. After 8-h incubation in MBM, oocytes were transferred to MBM containing [^{35}S]Met and [^{35}S]Cys and radiolabeled at 19°C for 72 h. The oocytes were then immunoprecipitated with anti-Ca^{2+}-ATPase, anti-Na^+/K^+-ATPase β-subunit or anti-MetAP2 antibodies. The immunoprecipitates were analyzed by SDS-PAGE and autoradiographed. The antibodies and cRNAs used are shown above each lane. *Arrowhead* indicates SR Ca^{2+}-ATPase

precipitated with anti-MetAP2 when oocytes were injected with SR Ca^{2+}-ATPase cRNA alone (Fig. 2, lane 2), consistent with an association between SR Ca^{2+}-ATPase and endogenous MetAP2 in oocytes.

The low-level coprecipitation of SR Ca^{2+}-ATPase with MetAP2 antisera suggested the possibility that the coprecipitate was an intermediate in the process leading to mature SR Ca^{2+}-ATPase. Therefore, to compare the duration of their association with that of the maturation process, we performed pulse-chase experiments.

Pulse-Chase

After labeling for 1 h in a medium containing radioactive amino acids, the oocytes injected with both SR Ca^{2+}-ATPase cRNA and MetAP2 cRNA were transferred to the chase medium for 1–12 h, extracted and immunoprecipitated with anti-SR Ca^{2+}-ATPase or anti-MetAP2 (Fig. 3). The SR Ca^{2+}-ATPase coprecipitated with anti-MetAP2 was chased out rapidly, declining to about 40% after a 12 h chase. In contrast, the amount of SR Ca^{2+}-ATPase precipitated with anti-SR Ca^{2+}-ATPase increased gradually.

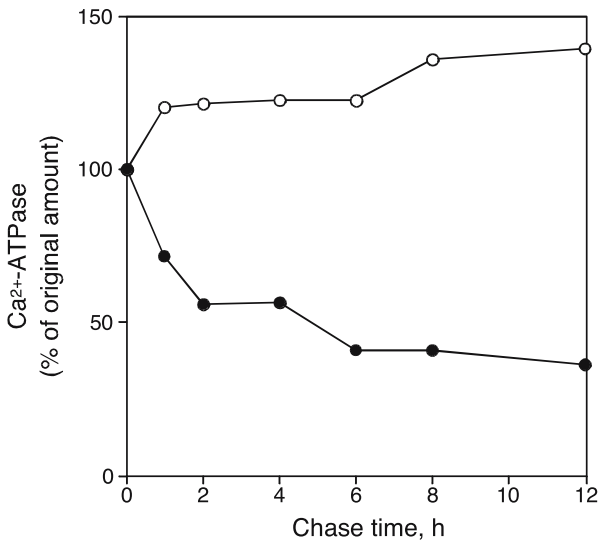

Fig. 3 Pulse-chase. SR Ca^{2+}-ATPase and MetAP2 cRNAs were injected into oocytes, which were incubated for 8 h, radiolabeled with [^{35}S]Met and [^{35}S]Cys for 1 h and transferred to the chase medium containing a 100-fold molar excess of unlabeled amino acids. At the indicated intervals, oocytes were quickly frozen in dry ice-ethanol. SR Ca^{2+}-ATPase in the oocytes was immunoprecipitated by either anti-SR Ca^{2+}-ATPase (*open circles*) or anti-MetAP2 (*closed circles*). The immunoprecipitates were analyzed by SDS-PAGE and the amounts of precipitated SR Ca^{2+}-ATPase quantified from the radioactivity measured with a Bioimage Analyzer. The figure shows one representative experiment

These data suggest a transient association between SR Ca^{2+}-ATPase and MetAP2, ending sometime during maturation of SR Ca^{2+}-ATPase, and implicate MetAP2 in the maturation process.

Functional Expression of SR Ca^{2+}-ATPase

We examined the correlation between the amount of MetAP2 cRNA injected and the production of functional SR Ca^{2+}-ATPase. *Xenopus* oocytes were injected with SR Ca^{2+}-ATPase cRNA together with varying amounts of MetAP2 cRNA. Figure 4 shows that the level of SR Ca^{2+}-ATPase precipitable with anti-SR Ca^{2+}-ATPase increased with increasing amounts of MetAP2 cRNA injected (Figs. 4A, B). The Ca^{2+}-ATPase activity in the microsomes from cRNA-injected oocytes was also elevated when MetAP2 cRNA was coinjected (Fig. 4C). These results indicate that MetAP2 facilitates the expression of functional SR Ca^{2+}-ATPase in a dose-dependent manner.

Prevention of SR Ca^{2+}-ATPase Synthesis by MetAP2-Specific Antiserum

As shown in Figure 2, anti-MetAP2 precipitates SR Ca^{2+}-ATPase in oocytes injected with SR Ca^{2+}-ATPase cRNA alone, indicating the existence of endogenous MetAP2 in

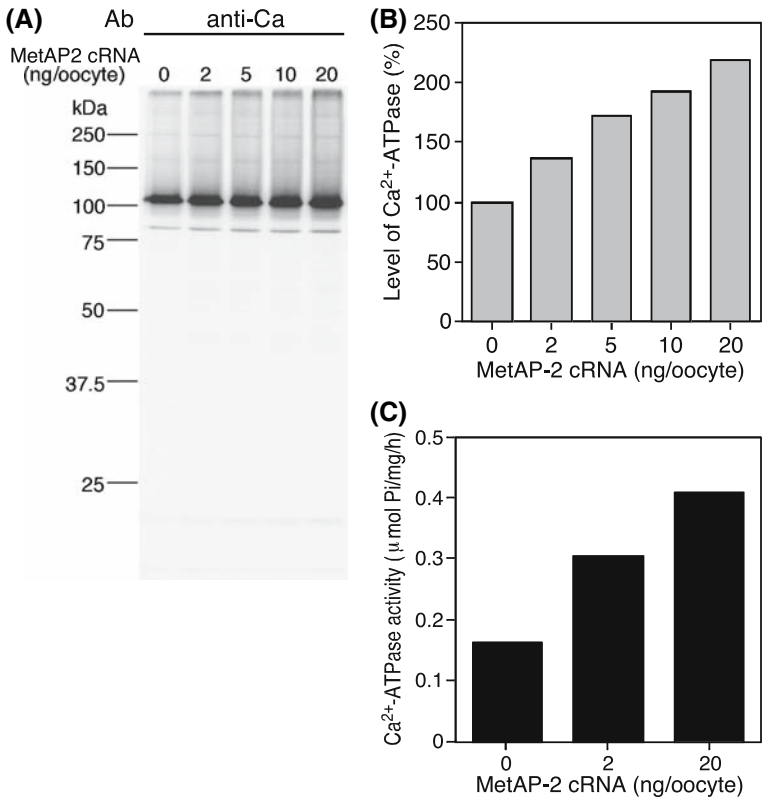

Fig. 4 The dose dependence of SR Ca^{2+}-ATPase expression on MetAP2 cRNA. SR Ca^{2+}-ATPase cRNA (10 ng in 23 nl water/oocyte) was injected with varying amounts of MetAP2 cRNA (0-20 ng in 23 nl/oocyte). (*A*) After radiolabeling for 72 h with [^{35}S]Met and [^{35}S]Cys, oocytes were immunoprecipitated with anti-SR Ca^{2+}-ATPase. (*B*) The bands of SR Ca^{2+}-ATPase in *A* were quantified as described in Materials and Methods. Averages of two independent experiments are shown. (*C*) After 72-h incubation, microsomes from the oocytes were assayed for Ca^{2+}-ATPase activity. The ATPase activity shown is an average of two independent experiments

oocytes (Fig. 2, lane 2). One model incorporating all these results has the endogenous MetAP2 acting as a molecular chaperone for SR Ca^{2+}-ATPase. In this model, the anti-MetAP2 would be expected to prevent the synthesis of SR Ca^{2+}-ATPase when added to oocytes containing injected SR Ca^{2+}-ATPase cRNA. As shown in Figure 5, the amount of SR Ca^{2+}-ATPase precipitated with anti-SR Ca^{2+}-ATPase decreased with increasing amounts of anti-MetAP2 injected into the oocytes (upper row). In the control, non-specific rabbit immunoglobulin G (IgG) had little, if any, effect on the synthesis of SR Ca^{2+}-ATPase (lower row).

Discussion

The β-subunit of the Na^+/K^+- and H^+/K^+-ATPases is absolutely required for the correct incorporation of the nascent α-subunit of the respective ATPases into membranes (Noguchi et al. 1987, 1990; Colonna et al. 1997; Beguin et al. 1998; Geering 2001). The question we address in this work is how SR Ca^{2+}-ATPase, lacking a β-subunit, folds correctly in the membrane.

In this work, we show that a cytoplasmic soluble protein, MetAP2, associates transiently with SR Ca^{2+}-ATPase and we hypothesize that MetAP2 acts as a molecular chaperone for the incorporation of nascent SR Ca^{2+}-ATPase into membranes. Our data suggest that once the incorporation is complete, the SR Ca^{2+}-ATPase releases MetAP2 and becomes functional. MetAP2 is a soluble cytoplasmic protein. In contrast, the β-subunit is a membrane protein that traverses the membrane once leaving a short N-terminal peptide (about 40 amino acids) on the cytoplasmic side. Since mAbHKβ cross-reacts with MetAP2 and thereby prevents the synthesis of SR Ca^{2+}-ATPase (Noguchi et al. 2003), the epitope for mAbHKβ should be in the short N-terminal peptide of the β-subunit and shared by MetAP2. The sequence GRT/S is conserved in the N-terminal sequence of the β-subunits and MetAP2 and serves as a candidate for this epitope. According to the manufacturer, mAbHKβ recognizes the N-terminal region spanning the GRT sequence.

MetAP2 catalyzes the cotranslational removal of the initiator methionine from the nascent polypeptide in eukaryotic cells (Bradshaw et al. 1998). In addition, it regulates protein synthesis by protecting the α-subunit of eukaryotic initiation factor 2 (eIF_2) from phosphorylation [protection of eIF2α phosphorylation (POEP) activity] (Datta 2000; Ray et al. 1992). The potent angiogenesis inhibitor fumagillin selectively and covalently binds to MetAP2 and blocks its aminopeptidase activity (Sin et al. 1997). We found that fumagillin does not affect the synthesis of SR Ca^{2+}-ATPase in oocytes (*data not shown*), indicating that the aminopeptidase activity is not involved in the molecular chaperone-like activity of MetAP2. Since

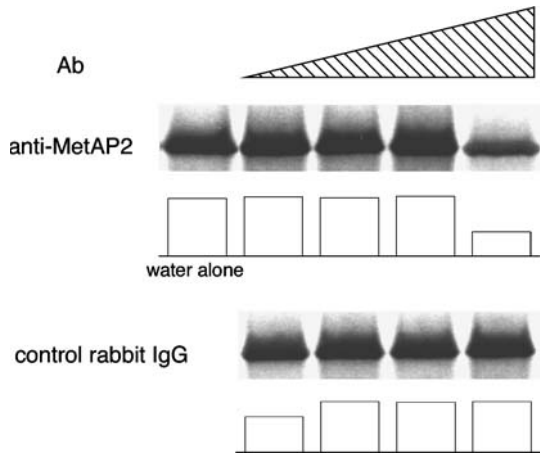

Fig. 5 Prevention of SR Ca^{2+}-ATPase synthesis with antibodies. Oocytes were injected with SR Ca^{2+}-ATPase cRNA, incubated for 8 h, injected with antibodies (23 nl/oocyte) of various dilutions (water alone, 1:1,000, 1:100, 1:10 and undiluted, from left to right) and incubated for 1 h. *Hatched triangles* indicate increasing concentration of antibody (Ab). The oocytes were then radiolabeled with [^{35}S]Met and [^{35}S]Cys for 1 h and the labeled oocytes frozen in dry ice-ethanol and immunoprecipitated with anti-SR Ca^{2+}-ATPase. For the control, SR Ca^{2+}-ATPase from water-injected oocytes (23 nl/oocyte) is shown at the left side of the anti-MetAP2 series. A series of oocytes injected with nonspecific rabbit IgG at similar dilutions (or undiluted) showed little or no inhibition of SR Ca^{2+}-ATPase synthesis (*lower row*). Each band was quantified as described in Materials and Methods, and the values relative to water alone are shown as bar diagrams. The experiment was repeated three times, and a representative result is shown

the involvement of POEP activity in the chaperone-like activity seems quite unlikely, the molecular chaperone activity would be the third function of MetAP2. In principle, MetAP2 could regulate Ca^{2+} concentration in muscle cells by affecting the mobilization of SR Ca^{2+}-ATPase.

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