Immonoaffinity Purification and Identification of the Molecular Chaperone Calnexin

Tetsuro YAMASHITA,† Emiko KIYOKI, Yasuhiro TOMITA, andHideharu TAIRA

Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

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We have developed a method for the immunoaffinity purification of calnexin, an endoplasmic reticulum molecular chaperone, and analyzed the molecular weight of purified calnexin using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF-MS). Calnexin was thereby found to have a molecular weight of 66.1 × 10^3, which is nearly identical to the molecular weight estimated from the protein sequence.

Key words: calnexin; molecular chaperone; affinity purification; mass spectrometry

Calnexin is a type I membrane-bound protein localized in the endoplasmic reticulum (ER) and is a component of molecular chaperones of the ER, including BiP (GRP78), calreticulin, and GRP94. Recent studies indicated that calnexin transiently associates with a number of newly synthesized glycoproteins, recognizing the partially trimmed monoglycosylated form of the N-linked oligosaccharides during their folding and maturation. Calnexin was first discovered in the canine rough ER fraction as a major phosphoprotein of 573 amino acids with an ER-localization signal (RKPRRE) at the carboxyl terminus, and no potential N-glycosylation sites according to the cDNA sequence. When analyzed by SDS-PAGE, however, it migrates with an apparent molecular mass of 88–90 kDa. The anomalous behavior of calnexin during the electrophoretic migration has been explained by its long stretches of acidic amino acids, but little information has been obtained about its post translational modifications except phosphorylation and N-glycosylation.

To analyze the molecular structure of calnexin, we developed a method for the purification of calnexin with immunoaffinity chromatography.

A polyclonal antibody against the C-terminal 14 amino acid residues of human calnexin (ILNRSPR-NRKPRRE) was prepared as described in Ref. 9. The antibody was purified with the peptide-conjugated AF-amino Toyopearl (Tosoh) matrix and then coupled to Protein A-Sepharose FF (Pharmacia) beads by using dimethylpimelidate according to the procedure described in Ref. 10.

Rabbit liver microsomes were prepared according to Ref. 9 for use as the source of calnexin. Rabbit liver was homogenized in 2 volumes (w/v) of 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM MgCl2 (STKM buffer) using a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 680 × g for 10 min at 4°C and the supernatant was then centrifuged at 100,000 × g for 1 h at 4°C. The supernatant was removed and the precipitate (microsomes) was suspended and homogenized in 150 mM Tris-HCl (pH 8.0) and centrifuged at 100,000 × g for 1 h. The precipitate was homogenized in STKM buffer at a protein concentration of 10–20 mg/mL.

The microsomes were solubilized in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 1% deoxycholate (RIPA buffer) at a protein concentration of 2 mg/mL. The solution (5–10 mL) was added to 0.5 ml of Protein A Sepharose FF for 1 h at 4°C with gentle mixing to remove the intrinsic IgG fraction present as a contaminant from the rabbit serum. The solubilized proteins were put on an anti-calnexin IgG-conjugated column (1.0 ml) pre-equilibrated with RIPA buffer at a flow rate of 0.1 ml/min at 4°C and the column was washed with 20 bed volumes of wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA).

In a preliminary experiment, the 90-kDa peptide adsorbed to the matrix was efficiently eluted from the column by 0.1 M Gly-HCl buffer at pH 2.5; however, the acid-eluted materials included small amounts of other proteins, presumably unconjugated IgG components and/or nonspecific binding proteins (data not shown). Alternatively, proteins associated with the column were eluted with 50 μg/ml of the synthetic peptide used as the antigen in the wash buffer at a flow rate of 0.1 ml/min and analyzed by SDS-PAGE. As shown in Fig. 1, a single peptide with an apparent molecular mass of 90 kDa was eluted from the immunoaffinity column as a relatively broad peak by the addition of the antigen peptide. The samples were electrophoresed onto the polyvinylidene difluoride (PVDF) membrane and N-terminal amino acid sequence was obtained by automated Edman degradation sequencer (PPSQ-21, Shimadzu Co., Ltd). The obtained N-terminal sequence, His-Asp-Gly, was identical with the canine calnexin.

The peptide eluted fraction (2 ml) was collected and concentrated to approximately 50 μl using a centrifugal concentrator (Ultra Free 4, Millipore Co.). One μl of the concentrated protein solution was spotted on a metal sample plate and mixed with an equal volume of matrix solution (10 mg/ml 3,5-dimethoxy-4-hydroxy cinnamic
acid (sinapinic acid, Aldrich) in 40% acetonitrile, 0.1% trifluoroacetic acid. The mixture was left to dry at room temperature and the MALDI mass spectrum was acquired on a PerSeptive Voyager RP biospectrometry with a 337 nm nitrogen laser. Figure 2 shows the mass spectrum, with a single peak at a molecular mass of 66.1 kDa, which is in good agreement with the molecular size estimated from the protein sequence of mammalian calnexin deduced from the cDNA sequence. This shows directly that the apparent large increase of molecular mass on SDS-PAGE analysis is not a result of post-translational modification.

In this study we developed a one-step procedure for the purification of calnexin, a membrane-bound molecular chaperone protein localized in the ER, using an immunoaffinity matrix conjugated with anti-peptide antibodies and a peptide-elution method. By adding a large molar excess of the synthetic peptide, calnexin was efficiently eluted from the matrix. Because the peptide elution is milder than pH-shift elution methods, which may denature the conjugated antibodies and the antigen proteins, this procedure may be useful for the purification of unstable proteins in the active form. Particularly for the studies of molecular chaperones, this method is expected to be useful in the isolation of chaperone molecules that remain associated with nascent peptides.

Recently, the MALDI TOF-MS technique has been developed for mass analysis of large biomolecules such as proteins and nucleic acids. In this experiment, the calculated average mass of 66.1 k corresponds well with the 65.4 k deduced from the cDNA sequence of canine or human calnexin, and the difference between these values may be due to the phosphorylation of the protein. The mass analysis for the dephosphorylated calnexin is under investigation. Further studies on the molecular structures of nascent polypeptides as well as chaperones can be done in combination of the immunoaffinity purification method and detailed mass analysis by MALDI TOF-MS.

References
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