Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 65-70

A CHICKEN TRANSFERRIN BINDING PROTEIN IS HEAT SHOCK PROTEIN 108

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A previously described transferrin binding protein from chicken oviduct has been identified as a heat shock protein, HSP108, by microsequencing of RP-HPLC purified tryptic peptides. The protein purified from oviduct by SDS-PAGE or from liver by ovotransferrin-affinity chromatography reacts with a monoclonal antibody raised against HSP108. The cDNA sequence predicts a KDEL peptide at the carboxyl terminus, but the protein does not react with anti-KDEL monoclonal antibodies, suggesting that HSP108 is processed in a way that eliminates the KDEL epitope.

We previously identified an estrogen-induced membrane glycoprotein from chicken oviduct that exhibits transferrin binding activity and shares a number of physical properties with the human transferrin receptor (1,2). Thus, the oviduct protein is a disulfide-linked homodimer with a subunit MW of about 100 KDa, is tightly membrane bound, and contains N-linked and O-linked oligosaccharides. In this report we show that the transferrin binding protein is a heat shock protein, HSP108. HSP108 was purified, cloned and sequenced by O'Malley and coworkers (3-5). It belongs to a family of heat shock proteins which contain a predicted carboxyl terminal KDEL sequence and are, therefore, presumptive RER residents (6). The functions of these proteins, especially HSP108, are not yet clearly defined (7,8). The data presented here also suggest that the KDEL sequence in HSP108 has been removed or modified.

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0006-291X/94 $5.00

MATERIALS AND METHODS

Protein Purification: Oviduct membranes were prepared as described (1,9). The oviduct transferrin binding protein was purified by SDS-PAGE and electroelution (10, cf.1). Briefly, oviduct membranes (0.5 mL, ca. 40 mg/ml protein) were solubilized and subjected to SDS-PAGE (11) on 3 mm thick 7.5% slab gels until the bromphenol blue had run off the gel. The gel was lightly stained with Coomassie blue (0.1% in 10% CH_3OH, 0.5% acetic acid) and destained in 10% CH_3OH. The desired band was excised and frozen. Pooled gel slices were electroeluted in Centricon-10 concentrators and the total volume was reduced to less than 1 mL. Aliquots of the protein were analyzed by SDS-PAGE. An estimate of the protein concentration was obtained by comparison of the staining intensity with known amounts of ovotransferrin.

The transferrin binding protein from chicken liver was affinity purified. Briefly, liver was homogenized in 0.25M sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM $MgCl_2$ (0.25M STM). The homogenate was filtered through several layers of gauze and centrifuged at 280 x g for 5 min. The supernatant was centrifuged at 1500 x g for 10 min. and the resulting pellet was suspended in 0.25M STM, solubilized with 1% Triton X-100, and passed over an ovotransferrin-AffiGel column (12). The protein was eluted in 2M KCl, 50 mM Hepes, pH 7.4, 0.1% Triton X-100, and concentrated using Centricon-30 concentrators.

Peptide Purification and Sequencing: Purified oviduct protein was precipitated with CH_3OH/CH_3Cl as described (13). The precipitate was dissolved in 0.5M Tris/HCl, pH 8.6, containing 2% SDS, reduced and carboxamidomethylated (14), precipitated and suspended in 8M urea. Trypsin digestion was performed as previously described (15) except that three one hour incubations were used (trypsin/protein 1:100, 1:200, 1:200). Peptides were separated by RP-HPLC on a Vydac C4 column using a linear gradient of 0 to 80% CH_3CN containing 0.1% TFA. Peptides of >2 KDa were first isolated in the void volume fraction of a BioGel P-2 column (0.9x89 cm) equilibrated in 0.1% TFA. When required, peptides were further purified on a Vydac C18 column. Peptides were sequenced by automated Edman degradation on an Applied Biosystems 470A Gas Phase Protein Sequencer. The GCG Sequence Analysis Software Package (16) was used to search the GenBank data base and align peptide sequences with the sequence of HSP108.

Western Blot Analysis: The transferrin binding protein was analyzed by western blots using a monoclonal antibody (9G10) raised against HSP108 (4) obtained from Stressgen, Inc., Vancouver, BC, Canada and two monoclonal antibodies (#'s 256 and 259) directed against KDEL peptides, obtained from Dr. Richard M. Napier, Horticultural Research Institute, Kent, England (17). Following SDS-PAGE, proteins were transferred to nitrocellulose and stained with 0.1% Ponceau S. Blots were probed with specific antibodies as described earlier (1) and stained with HRP-conjugated goat anti-rat IgG (Kierkegaard and Perry Labs, Gaithersberg, MD). High molecular weight markers were obtained from BioRad Laboratories.

RESULTS AND DISCUSSION

We have shown that an estrogen-induced membrane glycoprotein from chicken oviduct exhibits transferrin binding activity and is distinct from the embryonic chicken red blood cell transferrin receptor (1,18,19). To identify the oviduct protein we prepared tryptic peptides and subjected them to micro-sequence analysis. Fourteen individual peptides were sequenced and found to align with an oviduct heat shock protein, HSP108 (Fig. 1). HSP108 contains

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                                VDVDATVEEDE
  1 MKSAWALALACTLLLAASVTAEEVDVDATVEEDLGKSREGSRTDDEVVQREEEAIQLDGL   60

                     FAFQAEVNR                    ELISNASD
 61 NASQIKEIREKSEKFAFQAEVNRMMKLIINSLYKNKEIFLRELISNASDALDKIRLISLT  120

                          NMLHVTDTGI
121 DENALAGNEELTVKIKCDKEKNMLHVTDTGIGMTKEELIKNLGTIAKSGTSEFLNKMTEM  180

                                    HN-DTQHIWESDSNEF
181 QDDSQSTSELIGQFGVGFYSAFLVADRVIVTSKHNNDTQHIWESDSNEFSVIDDPRGNTL  240

                          ELDT          QFINFPIYV    TETVEEPVEE
241 GRGTTITLVLKEEASDYLELDTVKNLVKKYSQFINFPIYVWSSKTETVEEPVEEEEAKEE  300
301 KEETDDNEAAVEEEEEEKKPKTKKVEKTVWDWELMNDIKPIWQRPSKEVEEDEYKAFYKT  360
361 FSKEHDDPMAYIHFTAEGEVTFKSILFVPNSAPRGLFDEYGSKKSDFIKLYVRRVFITDD  420

                                                          YN
421 FHDMMPKYLNFVKGVVDSDDLPLNVSRETLQQHKLLKVIRKKLVRKTLDMIKKIAEEKYN  480

    DTF
481 DTFWKEFGTNVKLGVIEDHSNRTRLAKLLRFQSSHHESNLTSLDQYVERMKEKQDKIYFM  540

                              YEQVIYL
541 AGASRKEAESSPFVERLLKKGYE-VIYLTEPVDEYCIQALPEFDGKRFQNVAKEGVKFEES 600

          EALEKEFEPLLN
601 EKSKESREALEKEFEPLLNWMKDKALKDKIEKAVLSQRLTQSPCALVASQYGWSGNMERI  660

                                FEIN            VKENEDDK-V
661 MKAQAYQTGKDISTNYYASQKKTFEINPRHPLIKDMLRRVKENEDDKTVSDLAVVLFETA  720

       SGYMLPD
721 TLRSGYMLPDTKEYGDRIERMLRLSLNIDLDAKVEEEPEEPEDAAEEAEQDEEEVDADAE  780
781 DSETQKESTDVKDEL 795
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FIGURE 1. Alignment of tryptic peptide sequences with the peptide sequence of HSP108 derived from the cDNA. The peptide at position 24-34 was published earlier (1). The italicized peptide, 608-613, overlaps a second peptide from 613-619.

seven potential N-linked glycosylation sites and the oviduct protein possesses high mannose and complex neutral N-linked oligosaccharides (1). Three of the N-linked consensus sites were sequenced. The absence of an ASN residue in the peptide corresponding to ASN-216 suggests it is the location of an N-linked oligosaccharide. Similarly, the absence of THR-708 suggests it may also be modified, perhaps by an oligosaccharide or phosphate moiety.

Earlier studies revealed that the oviduct protein is also present in other tissues- most notably, liver, ovary, and brain (19). The identification of the oviduct transferrin binding protein as HSP108 permitted western blot analysis with a monoclonal antibody directed against HSP108. In order to extend our observations regarding the transferrin binding activity of HSP108 the protein was purified from chicken liver by affinity chromatography. As shown in figure 2, the protein obtained from liver reacts with the HSP108 monoclonal antibody. These results confirm that HSP108 possesses transferrin binding activity and demonstrate that the liver protein shares vital properties with the oviduct protein.

The apparent identity of the oviduct transferrin binding protein with HSP108 puts it into a family of heat shock proteins, which includes ERp99 (20), GRP94 (21,22), tumor rejection antigen gp96 (23,24), and HSP100 (25,26). All of these proteins, including HSP108 are predicted to contain KDEL sequences at the carboxyl terminus, implying retention in the RER (6). However, as we previously showed, oviduct HSP108 contains complex N-linked

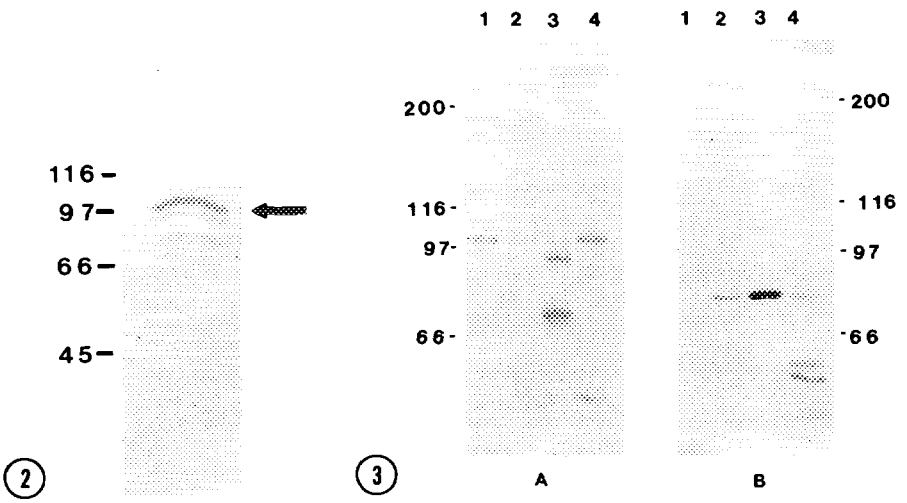

FIGURE 2. Western blot of affinity purified liver HSP108. Liver transferrin binding protein was purified on a transferrin-AffiGel column as described in Materials and Methods, the purified protein was subjected to analysis on an SDS-PAGE minigel and probed with antibody 9G10 directed against HSP108 at a dilution of 1/500. The position of HSP108 detected by Ponceau S is indicated by the arrow, along with the MW standards.

FIGURE 3. Western blots with monoclonal antibodies. An SDS-PAGE gel was run as described in Materials and Methods, cut in half and each half was stained with specific monoclonal antibodies. Panel A: The blot was probed with anti-HSP108 at a dilution of 1/500. Panel B: The blot was probed with an anti-KDEL monoclonal antibody (#256) at a dilution of 1/5. In each panel the lanes are- 1, purified oviduct protein (1 μg); 2, oviduct membranes (~150 μg); 3, chick liver membranes (~150μg); 4, rat liver membranes (~150 μg). The positions of MW markers are indicated.

and sialyl O-linked oligosaccharides (1), and such extensive carbohydrate processing suggests that HSP108 may not be restricted to the RER. Indeed, gp96 and endoplasmin have been shown to be present on the cell surface as well as the RER (24,27). Therefore, antibodies against the KDEL sequence were used to probe for the presence of KDEL in HSP108. As shown in figure 3A the purified oviduct protein as well as membrane preparations from oviduct, chick liver and rat liver react with the anti-HSP108 antibody. Conversely, HSP108 does not react with a KDEL monoclonal antibody (Fig. 3B). Identical results were obtained with the second antibody, #259. However, reactive proteins are observed in the membrane preparations at a MW of ~ 78 KDa, most likely BiP (GRP 78). It should also be noted that the affinity purified protein from liver does not react with the KDEL monoclonal antibody (data not shown). It appears, therefore, that HSP108 has been processed either to remove or to obscure the KDEL sequence predicted by the cDNA.

No clear function for this family of heat shock proteins has been established (7,8). However, a number of them bind Ca^{++} (7,8), gp96 has been implicated in antigen presentation and possesses ATPase activity (28), and HSP100 binds actin filaments and calmodulin (25).

Our observations establish that HSP108 isolated from oviduct (1) and liver has transferrin binding activity. Based on comparison of cDNA sequences HSP108 is clearly distinct from a chicken transferrin receptor cDNA cloned by Chan and coworkers (29,30), which is consistent with our earlier peptide map and immunologic studies (18,19). Furthermore, we have demonstrated that the embryonic red blood cell receptor is not immunologically detectable in other tissues (19), in agreement with the reported undetectable steady-state levels of chick red blood cell receptor mRNA in mature tissues (31). On the other hand, we have shown the presence of the "oviduct" transferrin binding protein in tissues with high iron requirements (19). The oviduct transferrin binding protein is a post-translationally modified form of HSP108 and the binding of transferrin by HSP108 implies a role for the protein in iron metabolism.

ACKNOWLEDGMENTS We thank Drs. I. Poola and D. D. Pless for preliminary peptide sequence data. This work was supported by NIH grants HD09046 and GM43111.

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