An estrogen inducible 104 kDa chaperone glycoprotein binds ferric iron containing proteins: a possible role in intracellular iron trafficking

Indra Poola*

Departments of Pharmacology and Biochemistry and Molecular Biology, Howard University School of Medicine, 520 W. Street, NW, Washington D.C. 20059, USA

Received 26 August 1997

Abstract We have previously described an estrogen inducible, intracellular, homodimeric membrane glycoprotein (subunit M_r 104 kDa) which is structurally related to 'chaperone' proteins (Poola, I. and Kiang J.G., J. Biol. Chem. 269 (1994) 21762–21769). In this report we describe a novel finding that the 104 kDa chaperone protein exhibits affinity for iron containing proteins such as transferrins from several species, human lactoferrin and microbial ferric binding protein (FBP). A single ferric ion in the above proteins appears to be sufficient for binding. It also binds to immobilized ferritin. However, it does not exhibit any affinity for apotransferrins, apolactoferrin, apoferritin and apoFBP. This is the first report of a chaperone protein that exhibits affinity for iron containing proteins.

Key words: Estrogen-inducible membrane glycoprotein; Intracellular iron trafficking; Transferrin; Lactoferrin; Microbial ferric binding protein; Chaperone protein

1. Introduction

We have previously described the purification and characterization of an estrogen inducible membrane glycoprotein (EIMG) from chicken oviduct membranes. It is an intracellular disulfide linked homodimeric protein with a subunit M_r of 104 kDa. A 116 kDa polypeptide which is structurally related to the 104 kDa subunit was also isolated. Amino acid sequence analysis of proteolytic peptides showed that EIMG is structurally related to the 'chaperone' (stress regulated) proteins, chicken heat shock protein 108 (hsp108), hamster glucose regulated proteins (hagrp94), human tumor rejection antigen (hTRAgp96), and mouse endoplasmic reticular protein (mERp99). All of these proteins are shown to be highly homologous to each other and to yeast hsp90. Stress induction experiments showed that EIMG is induced after a prolonged heat treatment [1,2]. The chaperone functions of glucose regulated proteins and tumor rejection antigens have been previously investigated. It was reported that glucose regulated proteins are involved in protein folding, similar to hsp70 [3], and the tumor rejection antigens are involved in presenting antigenic peptides to MHC class I molecules [4]. They are also implicated in protein folding [5,6]. However, no function has been described for hsp108 thus far.

In the present study, we report a novel finding that EIMG binds ferric iron loaded transferrins from several species, human lactoferrin and microbial ferric binding protein (FBP). We demonstrate here that ferric ions are essential for binding of EIMG to the above proteins. EIMG affinity for iron containing proteins, structural homologies with chaperone proteins and intracellular localization suggest that it is probably involved in intracellular trafficking of iron.

2. Materials and methods

Human transferrin, human lactoferrin, bovine transferrin, mouse transferrin, ovotransferrin, protease inhibitors, aprotinin, pepstatin, leupeptin, chymostatin and PMSF, concanavalin A-Sepharose 4B, human hemoglobin, chicken heart cytochrome *c*, horse ferritin and apoferritin, casein agarose, gelatin agarose, histone agarose, fetuin agarose, thyroglobin agarose, lysozyme agarose, immunoglobulin agarose and trypsin were obtained from Sigma Chemical Company. Affi-Gel 10 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were from Bio-Rad. Rabbit polyclonal antibodies against full length EIMG were available from previous studies [2]. Hen oviduct membranes were prepared as described [7]. They were first washed with 0.1 M citrate buffer pH 5.0, followed by 0.1 M glycine-NaOH buffer, pH 10.0 and finally with 50 mM Tris-HCl pH 7.6 in cold 4°C to diminish the endogenous ovotransferrin. FBP, isolated from *Haemophilus influenzae*, was kindly provided by Dr. Timothy Mietzner, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA.

2.1. Preparation of immobilized transferrins, lactoferrin, FBP, ferritins, hemoglobin and cytochrome c

Five milligrams of each of the above proteins were immobilized to 1 ml of Affi-Gel 10 according to the manufacturer's directions. Affi-Gel 10 transferrins, Affi-Gel 10 lactoferrin and Affi-Gel 10 FBP were first washed with 0.1 M citrate, pH 5.0 to remove all bound iron and were saturated with defined amounts of ferric iron according to a previously described procedure [8].

2.2. Preparation of COOH-terminal and NH_2-terminal lobes of human transferrin and their immobilization to Affi-Gel

Transferrins are monomeric molecules of M_r 80 kDa, composed of two homologous regions [9] which were shown to have originated by gene duplication [10]. Previous studies have shown that each lobe reversibly binds one molecule of ferric ion and that the two iron binding half molecules could be isolated by trypsinization of iron saturated transferrin [11]. In the present study, the COOH-terminal and NH_2-terminal lobes of human transferrin were isolated using the procedure described previously [12]. Briefly, iron saturated human transferrin (70 mg) was hydrolyzed with trypsin (4 mg) in 50 mM Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$ for 24 h at 37°C. It was chromatographed on a Sephadex-100 column to remove the undigested transferrin from the COOH-terminal and NH_2-terminal lobes. They were finally separated by affinity chromatography on concanavalin A-Sepharose 4B as described [12]. The separated fractions were concentrated in Centriplus (Amicon) filters and immobilized to Affi-Gel 10 as above.

2.3. Binding of EIMG to immobilized transferrins, lactoferrin and FBP

The following procedure was used to test the binding property of EIMG to transferrins of various species, human lactoferrin, and FBP: the washed hen oviduct membranes, which contain EIMG, were solubilized by mild sonication in 100 mM Tris-HCl pH 7.6, 2% Triton X-

*Fax: (1) (202) 806-5553/4453.

Abbreviations: EIMG, estrogen inducible membrane glycoprotein; FBP, ferric binding protein

PII S0014-5793(97)01183-6

100, 2 mM PMSF, and 2 μg/ml each of the protease inhibitors leupeptin, pepstatin and aprotinin. The undissolved material was removed by centrifugation in a refrigerated centrifuge at 10000×*g*. The clear supernatant (approximately 15 mg protein in 1 ml) was added to 1 ml each of Affi-Gel 10 transferrins, Affi-Gel 10 lactoferrin, and Affi-Gel 10 FBP and rotated for 30 min at 4°C. The gels were loaded to columns and each column was washed with 200 ml of the solubilization buffer to remove all unbound proteins. Iron from transferrin, lactoferrin and FBP columns was removed by washing with 50 ml of 0.1 M citrate buffer, pH 5.0, 0.2% Triton X-100 and protease inhibitors as above. Finally, bound EIMG was eluted from the affinity columns with 2 ml of 0.1 M glycine-NaOH buffer, pH 10.0, 5 mM EDTA, 0.2% Triton X-100, 50 μg/ml desferrioxamine and protease inhibitors as above. The eluate was adjusted to pH 7.0 and concentrated in an amicon microconcentrator to 100 μl and analyzed for EIMG by SDS-PAGE and Western blotting. All the chromatographic procedures were conducted in a cold room. To test the contribution of iron for binding, oviduct membranes were solubilized and chromatographed on immobilized apotransferrins, apolactoferrin, and apoFBP as above and in the presence of 10 mM EDTA and 50 μg/ml desferrioxamine to chelate trace amounts of iron in the buffer salts. Binding to immobilized ferric loaded and apo-human transferrin COOH-terminal and NH_2-terminal lobes was also conducted by the same procedures.

2.4. Binding of EIMG to hemoglobin, cytochrome c and ferritins

EIMG binding to these proteins was tested by the same procedure as above except that the citrate buffer wash was deleted. Binding to Affi-Gel 10 apoferritin was tested in the presence of 5 mM EDTA and 50 μg/ml desferrioxamine similar to the procedure described for Affi-Gel 10 apotransferrins.

2.5. Binding of EIMG to proteins which do not contain iron

To test whether EIMG shows any affinity for proteins which do not contain iron, oviduct membrane extract was chromatographed on the following immobilized proteins: casein agarose, gelatin agarose, histone agarose, fetuin agarose, thyroglobin agarose, lysozyme agarose and immunoglobulin agarose. The immobilized proteins were allowed to interact with the oviduct membrane extract, loaded onto columns and washed with solubilization buffer (200 ml). The gels were finally eluted with 0.1 M glycine-NaOH, pH 10.0, 0.2% Triton-X 100, and protease inhibitors as above. The eluates were concentrated and analyzed for the presence of EIMG by SDS-PAGE and Western blotting.

2.6. Electrophoresis and Western blotting

SDS-PAGE (12%) was conducted in a Bio-Rad slab gel apparatus as described by Laemmli [13]. Proteins were transblotted to nitrocellulose membranes as described by Towbin et al. [14]. Blocking and antibody treatments were performed as described [15]. A dilution of 1:10000 anti-EIMG antibodies was used to probe the blots. The antigen-antibody complexes were detected using a 1:7500 dilution of the horseradish peroxidase conjugated goat anti-rabbit IgG and development with the enhanced chemiluminescence (ECL, Amersham) detection system.

3. Results and discussion

Previously an estrogen inducible membrane glycoprotein was identified and characterized in the chicken oviduct. Protein sequence analysis showed that it is structurally related to stress regulated proteins. Preliminary experiments indicated that it binds transferrin molecules. This was unexpected in light of its structural homologies (more than 95%) to chaperone proteins and less than 5% amino acid sequence homology with the chicken transferrin receptor. We have undertaken the current study to understand the transferrin binding property of EIMG. In this report, we describe a novel finding that EIMG binds ferric ion containing proteins.

We used crude oviduct membrane extract which contains EIMG to test its binding property with immobilized diferric, monoferric, and apotransferrins, lactoferrin, microbial FBP, ferritin, hemoglobin and cytochrome *c*. The results of EIMG binding to diferric transferrins from several species and lactoferrin are presented in Fig. 1. As seen in Fig. 1, EIMG binds to immobilized diferric transferrins of chicken, human, bovine and mouse equally well (Fig. 1, lanes 1, 3, 5, and 7 respectively). We observed several very closely spaced bands on Western blots between M_r 95 kDa and 104 kDa and a minor band at 116 kDa. We have previously shown that both 116 kDa and 104 kDa species are proteolytically labile molecules and they give rise to several lower molecular weight species even in the presence of various protease inhibitors [2]. Therefore, the closely spaced species of EIMG observed here are possibly proteolytically degraded species. EIMG also bound to immobilized diferric human lactoferrin, an iron binding protein from milk, which is structurally related to transferrins (Fig. 1, lane 9). The primary structures of transferrins of several species and lactoferrin are elucidated. Comparison of the sequences of transferrins from different species and lactoferrins reveals that they share amino acid sequence homologies in the ferric binding region [16]. The affinity of EIMG to ferric loaded transferrins of several species and lactoferrin suggests that it recognizes the conserved amino acids in the ferric binding regions of transferrin and lactoferrin molecules. The transferrin and lactoferrin binding properties of EIMG described here are distinct from the ligand binding properties of the transferrin receptors. Unlike EIMG, transferrin receptors are species specific in that the receptors of one species do not bind the transferrins from a different species. Transferrin receptors also do not bind lactoferrin molecules [17].

To discern whether ferric ions or the conserved amino acids of transferrins are involved in the binding of EIMG, we conducted binding experiments with immobilized (1) human transferrin which has 30% and 100% saturated ferric ions and (2) apotransferrins and apolactoferrin. Our results show that the amount of EIMG bound was more than doubled when the ferric ion saturation increased from 30 to 100%

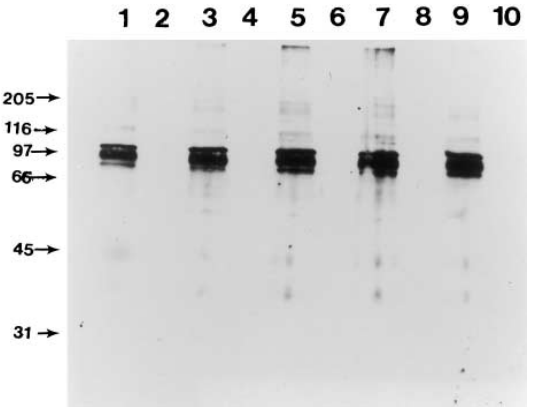

Fig. 1. Binding of estrogen inducible membrane glycoprotein to immobilized diferric and apotransferrins and lactoferrin. The chicken oviduct membranes were solubilized and chromatographed on diferric Affi-Gel 10 transferrins of various species, Affi-Gel 10 human lactoferrin, Affi-Gel 10 apotransferrins and Affi-Gel 10 human apolactoferrin according to the procedures described in Section 2. The affinity columns were eluted, the eluates concentrated and analyzed for EIMG by SDS-PAGE and Western blotting. Lanes 1, 3, 5, 7 and 9 contain the eluates from affinity gels of diferric transferrins from chicken, human, bovine, and mouse and human diferric lactoferrin respectively. Lanes 2, 4, 6 and 10 contain the eluates from immobilized apotransferrin affinity columns of chicken, human, bovine and mouse and human apolactoferrin, respectively.

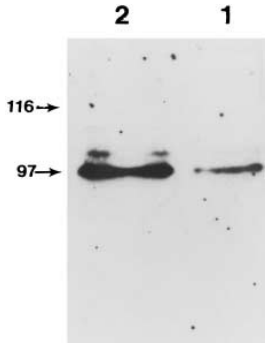

Fig. 2. Binding of estrogen inducible membrane glycoprotein to immobilized human transferrin loaded with different amounts of ferric ions. The chicken oviduct membranes were solubilized and chromatographed on 30% and 100% iron saturated human transferrin affinity columns according to the procedures described in Section 2. The affinity columns were eluted, the eluates concentrated and analyzed for EIMG by SDS-PAGE and Western blotting. Lane 1, eluate from immobilized human transferrin 30% with ferric iron; lane 2, eluate from immobilized human transferrin saturated 100% with ferric iron.

(Fig. 2, lanes 1 and 2 respectively), suggesting that ferric ions are involved in binding. This finding was further confirmed by the results obtained with apotransferrins. In sharp contrast to its high affinity for ferric transferrins, binding to immobilized apotransferrin and apolactoferrin affinity gels is totally absent (Fig. 1, lanes 2, 4, 6, 8, and 10). These findings clearly indicate that ferric ions are essential for the binding of EIMG to transferrins. After establishing that ferric ions are essential, we sought to determine whether two ferric ions are required or a single ferric ion is sufficient for binding. For this, we tested EIMG binding to immobilized monoferric COOH-, and NH_2-terminal lobes of human transferrin. It bound to immobilized monoferric human transferrin half molecules (data not shown), indicating that a single ferric ion is sufficient for binding. EIMG did not bind to immobilized apoCOOH and NH_2 lobes of human transferrin, consistent with earlier results.

The binding of EIMG to transferrins of various species and lactoferrin raised a question whether EIMG shows affinity for other ferric containing proteins which are not structurally related to transferrins or lactoferrins. To test the above, we conducted binding experiments with immobilized FBP isolated from the microbe *H. influenzae*. The primary structure, ferric binding property and function of FBP are well documented. It is a polypeptide of M_r 34 kDa, which reversibly binds a single ferric ion per molecule of protein with high affinity. The functional role of FBP was shown to be the transport of ferric molecules across the 'extracytoplasmic' periplasmic space of prokaryotes [18]. Although both FBP and transferrins reversibly bind ferric ions, they share little similarity in amino acid sequence. However, the amino acids involved in binding ferric ions in FBP appear to be remarkably similar to transferrins [19]. To test the ability of EIMG to bind FBP, oviduct membrane extract was chromatographed on immobilized monoferric FBP similar to transferrin affinity gels. The bound material was analyzed by SDS-PAGE and Western blotting. We detected EIMG (97 kDa–105 kDa and 116 kDa bands) in the FBP bound fraction similar to the pattern observed in binding experiments with diferric transferrins and lactoferrin (Fig. 1) (data not shown). It did not show any affinity to apoFBP.

The high affinity of EIMG observed for ferric containing proteins raised the question whether it has any affinity for other iron containing proteins which have irreversibly bound iron. For this, we conducted binding experiments with immobilized ferritin, hemoglobin and cytochrome *c*. Ferritin has irreversibly bound iron in the oxidized state. Hemoglobin and cytochrome *c* have bound heme to which ferrous ions are coordinated. Our results show that EIMG bound to immobilized ferritin but not to apoferritin, hemoglobin and cytochrome *c* (data not shown). In similar experiments, EIMG did not show any binding to immobilized non-iron containing proteins, casein-agarose, gelatin-agarose, fetuin-agarose, thyroglobin-agarose, lysozyme-agarose, histone-agarose and immunoglobulin-agarose. The observations that EIMG did not bind hemoglobin and cytochrome *c* suggest two possibilities: the iron should be (1) bound directly to the protein molecule to be recognized by EIMG or (2) in the ferric state. EIMG binding to non-heme, ferrous containing proteins could not be tested because of their unavailability.

The results described here are the first observations of a stress regulated protein exhibiting affinity for ferric containing proteins. The results presented above also indicate that EIMG is not specific to the protein molecule which carries the ferric iron since it binds transferrins of several species, lactoferrin, ferritin and FBP equally well. This is a novel observation since no other protein with such property has been described thus far. From the results above it appears that the ferric molecule is necessary for the binding between EIMG and the protein to which ferric iron is bound. It is not clear whether EIMG recognizes the ferric iron in the protein molecule or the conformation as modified by iron binding. The physiological implications of EIMG affinity for iron containing proteins are not clear. The intracellular location of EIMG excludes its participation in transferrin dependent, receptor mediated or transferrin independent iron uptake across the cell membrane. Iron uptake by the above two processes is described extensively [20–22]. However, the details of how ferric ions get transported intracellularly to target proteins are not known. Nunez et al. [23] have envisioned that it is an energy dependent, carrier mediated process through the transmembrane. However, there are no reports of such a transmembrane molecule which participates in the intracellular mobility of ferric ions until now. Since EIMG is an intracellular membrane protein possessing affinity for ferric iron containing proteins, it is conceivable that EIMG may be involved in facilitating the trafficking of ferric ions inside the cell.

Acknowledgements: This work was supported by grants from the National Cancer Institute (CA68986) and Ella O. Latham Charitable Trust. Dr. Timothy Meitzner is gratefully acknowledged for providing generous amounts of microbial ferric binding protein. The author is also thankful to Dr. Donna Williams for critical reading of the manuscript, and Dr. R.E. Taylor, chairman of the Pharmacology Department, for his enthusiastic support.

References

[1] Poola, I. and Lucas, J.J. (1988) J. Biol. Chem. 263, 19137–19146.
[2] Poola, I. and Kiang, J.G. (1994) J. Biol. Chem. 269, 21762–21769.
[3] Nigam, S.K., Goldberg, A.L., Ho, S., Rohde, M.F., Bush, K.T. and Sherman, S.K. (1994) J. Biol. Chem. 269, 1744–1749.
[4] Srivatsava, S.K. and Old, L.J. (1988) Immunol. Today 9, 78–83.

[5] Maki, R.G., Old, L.J. and Srivatsava, P.K. (1990) Proc. Natl. Acad. Sci. USA 87, 5658–5662.
[6] Li, Z. and Srivatsava, P.K. (1993) EMBO J. 12, 3143–3151.
[7] DeRosa, P.A. and Lucas, J.J. (1982) J. Biol. Chem. 257, 1017–1024.
[8] Graham, G. and Bates, G.W. (1976) J. Lab. Clin. Med. 88, 477–486.
[9] MacGillivray, R.T., Mendes, E., Schewale, J.G., Sinha, S.K., Lineback-Zins, J. and Brew, K. (1983) J. Biol. Chem. 258, 3543–3546.
[10] Park, I., Schaeffer, E., Sidoli, A., Baralle, F.E., Cohen, G.N. and Zakin, M.M. (1985) Proc. Natl. Acad. Sci. USA 82, 3149–3153.
[11] Evans, R.W. and Williams, J. (1978) Biochem. J. 173, 543–552.
[12] Alcantara, J., Yu, R.-H. and Schyvers, A.B. (1993) Mol. Microbiol. 8, 1135–1143.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad Sci. USA 76, 4350–4354.
[15] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[16] Baldwin, G.S. (1993) Comp. Biochem. Physiol. 106B, 203–218.
[17] Ismail, M. and Brock, J.H. (1993) J. Biol. Chem. 268, 21618–21625.
[18] Chen, C.-Y., Berish, S.A., Morse, S.A. and Meitzner, T.A. (1993) Mol. Microbiol. 10, 311–318.
[19] Nowalk, A.J., Tencza, S. and Mietzner, T.A. (1994) Biochemistry 33, 12769–12775.
[20] Kuhn, I.C., Schulman, H.M. and Ponka, P. (1990) in: Iron Transport and Storage (Ponka, P., Schulman, H.M. and Woodworth, R.C., Eds.), pp. 149–191. CRC Press, Boca Raton, FL.
[21] Kaplan, J., Jordan, I. and Sturrock, A. (1991) J. Biol. Chem. 266, 2997–3004.
[22] Oshiro, S., Nakajima, H., Markello, T., Kransnewich, D., Bernardini, I. and Gahl, W.A. (1993) J. Biol. Chem. 268, 21586–21591.
[23] Nunez, M.-T., Gaete, V., Watkins, J.A. and Glass, J. (1990) J. Biol. Chem. 265, 688–6692.