Calreticulin Binding Affinity for Glycosylated Laminin*

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Several lines of evidence indicate that calreticulin has lectin-like properties. As a molecular chaperone, calreticulin binds preferentially to nascent glycoproteins via their immature carbohydrates; this property closely resembles that seen for calnexin, a chaperone with extensive molecular identity to calreticulin. A cell surface form of calreticulin also exhibits lectin-like properties, binding specific oligomannosides including those covalently linked to laminin. In the present study we examined the interaction between calreticulin and laminin by means of surface plasmon resonance. The results show that calreticulin specifically binds to glycosylated laminin but fails to specifically bind tunicamycin-derived unglycosylated laminin or bovine serum albumin. Calreticulin binding to glycosylated laminin requires calcium and is abolished in the presence of EDTA. Scatchard analysis of binding yields an apparent association constant, $K_a$, of $2.1 \times 10^4 \text{M}^{-1}$ while kinetic analysis yields an estimate of the association rate, $(K_{assoc})$, as $2 \times 10^8 \text{M}^{-1} \text{s}^{-1}$. The composite results support calreticulin's lectin-like properties as well as its proposed role in laminin recognition, both in the cell interior and on the cell surface.

Calreticulin is found in many different locations in various eukaryotic cells, including the lumen of the endoplasmic reticulum (ER), the cell surface, perinuclear areas, and cytosolic granules (1). Some of these locations appear cell-specific, that is not all cells exhibit calreticulin at each location. The ER lumen is the most common location of calreticulin, a site where it is found in abundance (2). Given its strong avidity for calcium, calreticulin has been proposed to serve as a major calcium-sequestering protein within cells. Recently, calreticulin has been implicated as a molecular chaperone for nascent glycoproteins (3, 4); this activity resembles that of calnexin, a glycoprotein-selective chaperone whose domain structure substan-

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The abbreviations used are: ER, endoplasmic reticulum; BSA, bovine serum albumin.

1 Materials and Methods

Calreticulin was kindly provided by R. Freedman; it had been purified from bovine liver ER (2). Glycosylated laminin was purified from Engelbreth-Holm-Swarm tumor while unglycosylated laminin was purified from a mouse cell line incubated in tunicamycin (15). Tunicamycin-derived laminin lacks detectable N-linked carbohydrates, and its protein molecular structure appears virtually identical to glycosylated laminin (15).

2 Binding analysis of the interaction between calreticulin and laminin was performed on either a BIAcore or BIAcore 2000 biosensor (Pharmacia Biotech, Uppsala, Sweden) using contemporary technology (14). Experiments were performed at 25°C in 10 mM HEPES-buffered saline, 150 mM NaCl, and 0.005% surfactant P20 (Pharmacia) either with calcium (2 mM CaCl$_2$) or without (10 mM EDTA). Proteins were coupled to the sensor chip through free amino groups. The carbamoylated dextran surface (sensor chip CM5, Pharmacia) was first activated by addition of 0.2 mM N-ethyl-N'-(3-diethylaminopropyl)-carbo- diimide and 0.05 M N-hydroxsuccinimide (Pharmacia amine coupling kit), followed by addition of protein, either laminin, unglycosylated laminin, or bovine serum albumin (BSA), at a concentration of 20 µg/ml in 10 mM sodium acetate, pH 4.5. Remaining N-hydroxsuccinimide esters were blocked by the addition of 1.0 M ethanolamine hydrochloride, pH 8.5. Several different protein concentrations were immobilized in order to optimize conditions. In the experiments shown immobilization conditions were controlled such that all three proteins gave approximately 3000 resonance units of immobilized material.

3 Results

1 Immobilization of Laminin—The interaction between calreticulin and the glycosylated and unglycosylated forms of laminin was analyzed by the binding of soluble calreticulin to immobilized laminin. Immobilization of laminin was accomplished by coupling through amine groups, and successful immobilization was confirmed by the binding of an anti-laminin antiserum (data not shown).

2 Affinity of the Calreticulin–Laminin Interaction—The affinity of calreticulin binding to laminin was determined by equilibrium binding analysis on the BIAcore as has been performed previously in other systems (16, 17). A range of concentrations of calreticulin was injected over the immobilized surfaces of glycosylated laminin (Fig. 1, top), unglycosylated laminin (Fig.

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Calreticulin binds glycosylated laminin

1, middle, and, as a control cell, BSA (Fig. 1, bottom). The fast off rate of the interaction allows the binding to reach equilibrium in a very short time; therefore short injection times were used, and very little time was required between injections for the response to return to baseline levels. The signal increase observed in the BSA control cell appears to be due to refractive index changes, and this nonspecific response was subtracted from laminin flow cells to yield true binding responses. A plot of these data is shown in Fig. 2. Binding of calreticulin to glycosylated laminin demonstrates concentration dependence and saturability. A Scatchard plot of these data is linear and gives an association constant ($K_a$) of $2.4 \times 10^6$ M$^{-1}$ (Fig. 3). Three independent experiments gave a $K_a$ of $2.1 \pm 0.9 \times 10^6$ M$^{-1}$.

In contrast with binding to glycosylated laminin, binding of calreticulin to unglycosylated laminin showed very weak affinity, typically less than 5% of that seen for the glycosylated protein. These data indicate that calreticulin binding to laminin is dependent on carbohydrate.

Calcium is required for the interaction with glycosylated laminin—Because calreticulin has been identified as a calcium binding protein (18) we asked whether calcium was required for binding to glycosylated laminin. To test this a chelating agent was added to the calreticulin sample, and binding was tested in buffer lacking calcium (10 mM HEPES, 150 mM NaCl, 10 mM EDTA, and 0.005% surfactant P20). The calcium-free calreticulin was injected over the three protein surfaces, and binding responses are shown in Fig. 4. In the absence of calcium no significant binding is observed between calreticulin and laminin.

Analysis of the Kinetics—The off rate for this interaction is too fast to measure accurately using this system. The dissociation rate is clearly faster than 0.1 s$^{-1}$. So if one assumes a $K_{	ext{diss}}$ of 0.1 s$^{-1}$ and the association constant of $2 \times 10^6$ M$^{-1}$, then one can calculate an on rate ($K_{	ext{assoc}}$) of $2 \times 10^5$ M$^{-1}$ s$^{-1}$. This is the lowest possible estimate; it is probably faster than this. This analysis is consistent with observations that fast-on, fast-
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DISCUSSION

The objective of this study was to further explore interactions between calreticulin and the N-linked carbohydrates of laminin. Conceivably, intralumenal glycosylated laminin chains and/or assembled laminin molecules may transiently bind to molecular chaperones, including those which recognize glycoproteins. The present results provide a firm basis for potential ER intralumenal binding of glycosylated laminin molecules to calreticulin. Presumably, individual laminin subunits bind to calreticulin, followed by laminin molecular assembly, perhaps while the subunits are still complexed to the chaperone. Notably, laminin synthesized in the presence of tunicamycin fails to be secreted (15), perhaps reflecting the inability of certain chaperones such as calreticulin to properly interact with the unglycosylated protein.

Lectins require a suitable cation, often calcium, for sustaining their carbohydrate binding properties. Oligomannosides have been specifically implicated in cell surface calreticulin binding to laminin (7). The present results bolster the interpretation that calreticulin has lectin-like activity by demonstrating that it binds to glycosylated laminin in the presence of calcium while EDTA abolishes such binding. Calreticulin fails to bind to unglycosylated laminin, further substantiating its lectin-like properties. Intralumenal calreticulin binds nascent transferrin (4), a glycoprotein, and appears to interact with nascent myeloperoxidase via that glycoprotein’s N-linked oligomannosides (3), thereby resembling the binding of calnexin to nascent glycoproteins (20). Presumably, both intralumenal calreticulin and calnexin rely upon calcium to support their lectin-like activities.

Binding of carbohydrate ligands to both plant (24) and animal lectins (25) has been evaluated by surface plasmon resonance. Association on rates ranging from $1.63 \times 10^4$ to $5.7 \times 10^8$ M$^{-1}$ s$^{-1}$ were found, and association constants ranging from $6.2 \times 10^7$ to $4.3 \times 10^8$ M$^{-1}$ were reported. Our results for calreticulin binding to glycosylated laminin yield an on rate and association constant, which differ from those values, perhaps reflecting biological variation between various lectins and their ligands. Given the disparate molecular sizes of calreticulin (43 kDa) and laminin (about 900 kDa) and the magnitude of the sensorgram signals, which reflect their binding, more than one calreticulin molecule may bind each laminin molecule. Additional studies will be needed to quantitatively substantiate this interpretation.

In mouse melanoma cells the calreticulin-laminin complex itself may reach the cell surface, accounting for calreticulin appearance on the surface (9) and consistent with the observation that these cells produce and release laminin (21). A precedent for postulating such a pathway is that intraluminal calnexin, complexed to antigen receptor proteins, reaches the surface of immature thymocytes (22). This complex transits to the thymocyte surface from the ER, due to impairment of internal recycling of calnexin. The authors speculate that surface calnexin may mediate cell-cell lectin-dependent interactions and may also generate intracellular signals. It is already clear that surface calreticulin recognizes laminin (9) and fibrinogen (23); such recognition leads to specific cellular responses in each instance. Mouse melanoma cells, adherent to laminin, will spread once their surface calreticulin binds to a suitable oligomannoside (7). Human fetal fibroblasts bind the fibrinogen Bβ chain via surface calreticulin, thereby stimulating cell proliferation (23). Thus, cell signaling may be mediated by a new class of cell surface receptors, those which have lectin-like properties.

These studies measured the binding affinity of bovine ER-derived calreticulin and murine laminin. Given that calreticulin structure is highly conserved (18) and that the oligomannoside N-linked structures are virtually species-independent, it is not surprising that cross-species binding occurs. At present, it appears that intracellular and cell surface calreticulins may recognize similar carbohydrate structures, but direct comparison of these two proteins will be required to precisely define their ligand affinities. Interestingly, calreticulin, anchored in the ER membrane by a genetically engineered calnexin transmembrane domain, behaved more like calnexin than did the native non-anchored form of calreticulin (4). The anchored calreticulin efficiently bound the same spectrum of nascent glycoproteins as calnexin, while the non-anchored calreticulin did so with far less efficiency. Binding of both forms of calreticulin required the presence of appropriate oligosaccharide structures on the nascent glycoproteins. By analogy, we speculate that cell surface calreticulin of mouse melanoma cells, which we find cannot be removed by exhaustive washing and is therefore retained at the surface membrane, will differ in lectin binding from intraluminal calreticulin. Studies are in progress to investigate this possibility.

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