Calnexin Fails to Associate with Substrate Proteins in Glucosidase-deficient Cell Lines*

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Increasing evidence shows that calnexin, a membrane-bound chaperone in the endoplasmic reticulum, is a lectin that binds to newly synthesized glycoproteins that have partially trimmed N-linked oligosaccharides. It specifically attaches to core glycans from which two glucoses have been removed by glucosidases I and II. Several recent reports suggest, however, that it can also bind to proteins devoid of N-linked glycans. To investigate the extent of glycan-independent binding, we have analyzed two mutant cell lines (Lec 23 and PhaR2.7) that are unable to process the core glycans because they lack glucosidase I or glucosidase II, respectively. In contrast to parental cell lines, calnexin binding of substrate proteins was found to be virtually nonexistent in these cells. Neither cellular nor viral proteins associated with the chaperone. It was concluded that glycans are crucial for calnexin association and that the vast majority of substrate proteins are therefore glycoproteins.

The lumen of the endoplasmic reticulum (ER) contains a large number of chaperones and folding enzymes (see Refs. 1 and 2). They assist the folding, oligomeric assembly, and quality control of newly synthesized proteins, the majority of which are glycoproteins. Some of these folding factors, such as BIP, GRP78 and GRP94, are members of classical chaperone families while others are unique to the ER. The latter include calnexin, a membrane-bound protein that interacts transiently with a variety of soluble and membrane-bound substrate molecules (see Ref. 3).

Our studies have indicated that calnexin differs from previously characterized chaperones in being a lectin; it binds to glycoproteins with partially trimmed N-linked oligosaccharides (4, 5). The specificity for monoglucosylated chains (GlcMan\_\_\_\_\_GlcNAc\_\_) was recently confirmed by Ware et al. (32) using purified N-linked oligosaccharides. These results are significant because they link the folding of glycoproteins and their quality control in the ER directly to the process of oligosaccharide trimming. Based on these observations, we have proposed that the de- and reglucosylation cycle, known to operate in the lumen of the ER (6), is connected to calnexin binding and that the cycle plays a central role in the maturation of newly synthesized glycoproteins (4, 7).

Recent studies have confirmed the requirement for glycans in substrate binding to calnexin. These rely on glycosylation inhibitors (tunicamycin), α-glucosidase inhibitors castanospermine (CST) and 1-deoxynojirimycin, and mutagenesis to remove consensus glycosylation sequences (8–10). It has also been reported, however, that polypeptide chains that have no N-linked oligosaccharides can associate with calnexin. The latter observations include the redistribution of the unglycosylated CD3ε subunit of the T cell receptor in cells expressing a mutant version of calnexin, and the co-immunoprecipitation of unglycosylated thyroglobulin, class I and II major histocompatibility complex subunits and P-protein with calnexin (11–15). It has therefore been suggested that calnexin binding may occur in more than one way and may not require the presence of sugars.

To address the mode of substrate association with calnexin, we have analyzed mutant cells with glucosidase defects. Since they fail to remove glucoses from the core oligosaccharide, the glycoproteins do not reach the monoglucosylated form and are therefore unable to bind to calnexin via the lectin binding sites. Hence, these cells provide an opportunity to determine whether glycan-independent binding does take place under physiological conditions. Tests can be made without resorting to inhibitors and overexpression systems.

MATERIALS AND METHODS

Cell Lines, Viruses, and Reagents—Chinese hamster ovary (CHO) cells were grown in α-MEM with 8% fetal calf serum (R B. Bionetics, Inc., San Francisco, CA). One-third of the lysates were used for co-immunoprecipitation of glycosylated thyroglobulin, class I and II major histocompatibility complex subunits and P-protein with calnexin (11–15). It has therefore been suggested that calnexin binding may occur in more than one way and may not require the presence of sugars.

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The abbreviations used are: ER, endoplasmic reticulum; CST, castanospermine; GRP, glucose-regulated protein; BIP, immunoglobulin heavy chain-binding protein; CHO, Chinese hamster ovary cell line; Lec 23, glucosidase I-deficient cell line; PhaR2.7, glucosidase II-deficient cell line; BW 5147, mouse lymphoma cell line; VS, vesicular stomatitis virus; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HA, influenza hemagglutinin.

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PhaR 2.7.Lec23 are mutant CHO cells defective in glucosidase I. Cells were incubated at 37°C for 2 h to allow them to attach and form monolayers. 0.25 ml of serum-free medium were added to each well of a 24-well plate. Cell culture was performed in serum-free medium (J. T. Baker Inc., Phillipsburg, NJ) in serum-free and parent cell lines.

Chamber for 30 min with 15 μl of anti-calnexin antibody diluted 1:100 in medium A. The coverslips were washed twice with serum-free medium, and cells were permeabilized for 10 min at room temperature in 0.05% saponin (Sigma) in α-MEM containing 10 mM glycine (Sigma) and 10% horse serum (medium A) (Gemini Biocorporation Inc., Calabasas, CA). The coverslips were incubated in a humid chamber for 30 min with 15 μl of anti-calnexin antibody diluted 1:100 in medium A. After incubation, they were washed three times each for 5 min in medium A. The above incubation was repeated with fluorescein isothiocyanate-labeled anti-rabbit antibody.

RESULTS AND DISCUSSION

The two mutant cell lines used in this study were Lec 23 and PhaR 2.7. Lec 23 are mutant CHO cells defective in glucosidase I (23), and PhaR 2.7 cells are mutants of the BW 5147 mouse lymphoma cell line defective in glucosidase II (24). Previous work has established that glycoproteins are synthesized in both mutant cell lines, but glucose trimming is defective. Both mutant cell lines grow well in culture and synthesize normal or slightly increased amounts of calnexin compared with the parental lines (see below). Indirect immunofluorescence microscopy using anti-calnexin antibodies showed that the calnexin is distributed in a typical reticular ER pattern with nuclear rim staining (Fig. 1). The distribution of calnexin was identical to that seen in the parental cells.

To analyze the interaction of newly synthesized cellular proteins with calnexin in Lec 23 cells and in the parental CHO cells, monolayers were pulse-labeled for 10 min with 35S-labeled ProMix. CST was included in some of the cultures to prevent the action of glucosidases I and II. A CHAPS-solubilized lysate was prepared and immunoprecipitated using anti-calnexin antibodies, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

As reported previously (8), several labeled protein bands were precipitated with the anti-calnexin antibodies from labeled wild-type CHO cells (Fig. 2, lane 1). The band migrating at 90 kDa corresponded to calnexin itself, whereas the others were coprecipitating substrate proteins. When labeling was performed in the presence of CST (lane 2), only the calnexin band was seen, confirming the requirement for glucose trimming and the specificity of the antibodies.

In Lec 23 cells, the coprecipitation of proteins was dramatically reduced (lane 3). Few if any substrate proteins associated with calnexin. The background was, however, further suppressed by the addition of CST (lane 4), suggesting that it was specific. The presence of small background staining was consistent with the report that Lec 23 cells are somewhat leaky, i.e., the glucosidase I activity is not completely inhibited (23).

In infected wild-type CHO cells, we have previously reported efficient binding of two viral glycoproteins, influenza HA and VSV G protein, to calnexin (4, 8). This is also seen in lanes 5 and 9 (Fig. 2). Binding of these viral membrane proteins was blocked if they were synthesized in the presence of CST (lanes 6 and 10).

When Lec 23 cells were infected with the same viruses, calnexin binding of the two glycoproteins was virtually non-existent (lanes 7 and 8 and 11 and 12). That the cells were indeed infected is shown in Fig. 3. The amounts of labeled HA and G protein after immunoprecipitation with anti-HA and G-protein antibodies show that the amounts were comparable with or without CST (compare lanes 1 and 3 and 5 and 6 and lanes 2 and 4 and 7 and 8). As expected, the apparent molecular weights of HA and G protein were slightly higher in Lec 23 cells due to the lack of glucose trimming.

With the glucosidase II-deficient PhaR 2.7 cells, the results were similar to those seen for Lec 23. Whereas numerous newly synthesized proteins coprecipitated with anti-calnexin from the parental BW 5147 cells (Fig. 4, lane 3), virtually no labeled bands (except for calnexin itself) were seen in the PhaR 2.7 cells (lane 4). After infection of the wild type cells with VSV, the G protein was communoprecipitated with anti-calnexin as expected (lane 1), whereas coprecipitation from the mutant cells was virtually undetectable (lane 2). That the cells were infected, and that they expressed VSV G protein, was shown by immunoprecipitation using anti-G protein antibodies (Fig. 1, lanes 9 and 10). Both the membrane-bound, intact G protein (Gm) and the cleaved (Gs) forms were seen. Similar experiments were attempted with influenza virus, but being lymphoid, these cells could not be efficiently infected by this virus.

Taken together, the results showed that the majority, if not all, of the substrate proteins that normally associate with cal-
that fail to digimerize also often end up in large aggregates. It has, moreover, been our experience that transient expression of wild type proteins using the T7 vaccinia virus expression system (29) frequently results in aggregated products (27). When the substrate proteins are aggregated, it is difficult to know whether the communoprecipitation of a specific nonglycosylated substrate protein with calnexin occurs because of a direct contact between the two proteins or due to indirect association mediated by other proteins. Further studies using isolated proteins in vitro are needed to determine the various modes of calnexin binding to its substrate.

That Lec 23 and PhaR 2.7 cells are viable is interesting in view of their glucosidase defects and the lack of calnexin-mediated folding. These cells have been selected for growth and may therefore contain higher amounts of other folding factors. It is known that many glycoproteins can fold correctly, albeit usually less rapidly and less efficiently, in cells treated with glucosidase inhibitors (see Refs. 30 and 31). Evidently, they can fold and reach the mature conformation using other chaperones and folding enzymes.

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