Calreticulin Interacts with Newly Synthesized Human Immunodeficiency Virus Type 1 Envelope Glycoprotein, Suggesting a Chaperone Function Similar to That of Calnexin*

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The ubiquitous eukaryotic protein calreticulin has been detected in a wide variety of different cell types. Recently, calreticulin was found to bind in vitro to a number of proteins isolated from the endoplasmic reticulum. In addition, calreticulin has sequence similarities with the molecular chaperone calnexin. These data suggest that calreticulin might also act as a chaperone. We found that calreticulin associated transiently with a large number of newly synthesized cellular proteins. In cells expressing recombinant human immunodeficiency virus (HIV) envelope glycoprotein, gp160 bound transiently to calreticulin with a peak at 10 min after its synthesis. Binding of gp120 to calreticulin was not detected because proteolytic cleavage of gp160 occurs in the trans-Golgi. Nonglycosylated HIV envelope protein was not associated with calreticulin, suggesting a requirement for N-linked oligosaccharides on newly synthesized proteins as has been reported for calnexin. The in vivo binding kinetics of calnexin and calreticulin to gp160 were very similar. Sequential immunoprecipitations provided evidence for the existence of ternary complexes of gp160, calreticulin, and calnexin. The data suggested that most of the gp160 associated with calreticulin was also bound to calnexin but that only a portion of the gp160 associated with calnexin was also bound to calreticulin.

Highly conserved cDNAs encoding calreticulin, an abundant protein that has been localized to the lumen of the endoplasmic reticulum (ER),1 have been isolated from human, mouse, rabbit, rat, Xenopus, Aplysia, Drosophila, Oncorhynchus, Caenorhabditis, and Schistosoma libraries (for a review see Ref. 1). There is over 90% amino acid sequence identity between rabbit, mouse, human, and rat calreticulins (2). Calreticulin is considered to be the major Ca2+-binding protein in the nonmuscle ER (2). Other Ca2+-binding proteins in the lumen of this membrane system include endoplasmic (GRP94), immunoglobulin-binding protein (BiP or GRP78), and protein disulfide isomerase. As with calreticulin, these three proteins contain the C-terminal ER retention sequence KDEL preceded by clusters of acidic residues. A role for Ca2+ in the interaction of KDEL proteins with the KDEL receptor has been proposed (3, 4). There is evidence that the four ER proteins might have a similar transcriptional regulation (5). Because BiP, GRP94, and protein disulfide isomerase are believed to be involved in protein transport, folding, or assembly (6), a similar role has been considered for calreticulin. Calreticulin can be induced by amino acid deprivation (7) and heat shock (8), suggesting that calreticulin, like many chaperones, is a stress protein. In addition, several groups have reported that calreticulin binds to a number of proteins isolated from the ER (9–11). Also, calreticulin has sequence similarities with calnexin, a known molecular chaperone.

In accordance with these data, a recent study provided evidence that calreticulin serves as a chaperone in the biosynthesis of myeloperoxidase (12). Here, we show that calreticulin and calnexin bind transiently to glycosylated forms of the integral membrane protein encoded by the human immunodeficiency virus type 1 (HIV-1). Sequential immunoprecipitations suggest the existence of ternary complexes containing calreticulin, calnexin, and the HIV-1 envelope protein.

MATERIALS AND METHODS

Viruses and Reagents—Recombinant vaccinia virus vPE16, which expresses the HIV-1 env gene from a clone of the BH8 isolate (13), has been described previously (14, 15). The truncated proteins derived from HIV-1 gp160 (15) or HIV type 2 gp160 (16) were also expressed by recombinant vaccinia viruses. Tunicamycin and castanospermine were obtained from Boehringer Mannheim. 1-Deoxyxylulose, 1-deoxymannojirimycin, and bromodeoxyuridil were purchased from Oxford GlycoSystems (Bedford, MA).

Vaccinia Virus Infections, Pulse Labeling, and Immunoprecipitations—BS-C-1 cells were infected with 10 plaque forming units/cell. At 16 h postinfection, cells were lysed with 1% Triton X-100 in 150 mM NaCl, 10 mM iodoacetamide, 50 mM Tris (pH 7.4), incubated on ice for 10 min, centrifuged for 10 min, and stored at -80°C.

SDS-PAGE and Scanning Densitometry—Samples were prepared in Laemmli buffer with 1% SDS and 1% mercaptoethanol. Proteins were separated on 10% polyacrylamide gels and stained with Coomassie blue. Intensities of bands were measured with a PhosphoImager from Molecular Dynamics (Sunnyvale, CA).

Western Blotting—Proteins from extracts of vaccinia virus-infected cells expressing HIV-1 gp160 or HIV-2 gp120 were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with rabbit polyclonal serum 2144, raised against the truncated HIV envelope protein gp140, was used as a positive control (kindly provided by C. Broder). Calreticulin-directed serum was purchased from Affinity BioReagents (Neshanic Station, NJ). Serum directed to the C terminus of calnexin was obtained from StressGen (Victoria, Canada).

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1 The abbreviations used are: ER, endoplasmic reticulum; BiP, immunoglobulin heavy chain-binding protein; HIV, human immunodeficiency virus; HIV-1, HIV type 1; VSV, vesicular stomatitis virus; PAGE, polyacrylamide gel electrophoresis.

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‡ Supported by a fellowship from the German Bundesministerium ftir Forschung und Technologie, Stipendienprogramm Infektionskrankheiten.

(Received for publication, August 2, 1995, and in revised form, October 21, 1995)
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FIG. 1. Association of newly synthesized cellular proteins with calreticulin. BS-C-1 cells were metabolically labeled with [35S]methionine for 5 min and chased for the times indicated at the top of the figure. Then extracts of the cells were incubated with calreticulin-directed antiserum and protein A-Sepharose beads. The washed beads were treated with SDS and analyzed by SDS-PAGE and autoradiography. The arrow indicates calreticulin.

RESULTS

Calreticulin Associates Transiently with Newly Synthesized Cellular Proteins—Prior to studying the specific interaction of calreticulin with the HIV-1 envelope glycoprotein, we wished to determine if calreticulin associates transiently with polypeptides of the host cell. For this purpose, BS-C-1 cells were biosynthetically labeled for 5 min with [35S]methionine and chased for up to 120 min. Extracts from these cells were incubated successively with calreticulin-directed antiserum and protein A beads. As determined by SDS-PAGE, a large number of different proteins complexed with calreticulin, which has a mass of 60 kDa (9). The most protein was coprecipitated at 5 min after the pulse, and progressively less protein was coprecipitated with longer chase times (Fig. 1). The chaperone calnexin showed similar transient binding to newly synthesized cellular proteins (17). Because calreticulin and calnexin share some highly conserved amino acid sequences (1), we needed to exclude cross-reactivity of the anti-calreticulin serum with calnexin. For this purpose, lysates of BS-C-1 cells that had been labeled overnight with [35S]methionine were used for immunoprecipitations. The calnexin- and calreticulin-directed sera immunoprecipitated major proteins corresponding to the expected sizes of their target polypeptides as well as minor proteins that were presumably complexed with the chaperones (Fig. 2, lanes 1 and 2). To remove the complexed proteins, the protein A-Sepharose beads were incubated in SDS. Then the supernatants were used for a second round of immunoprecipitation by the same antibodies. As shown, calnexin and calreticulin were specifically immunoprecipitated, and no cross-reactivity of these two sera was detectable (Fig. 2, lanes 3 and 4).

HIV-1 Envelope Protein Binds Transiently to Calreticulin Early during Its Processing—To investigate if calreticulin like calnexin binds transiently to newly synthesized secretory glycoproteins, we used the well studied HIV-1 envelope glycoprotein gp160 expressed by the recombinant vaccinia virus vPE16 (15). The envelope protein is synthesized as a 160-kDa glycosylated precursor and is subsequently cleaved to gp120 and gp41 subunits in the Golgi. gp160 is processed rather slowly, especially in comparison with the influenza hemagglutinin and vesicular stomatitis virus G protein (18), which is an advantage for the study of transient chaperone-binding. The calreticulin-directed serum coprecipitated gp160 from infected cells as determined by Western blotting (Fig. 3, lane 2). By contrast, when a gp160-directed serum was used, both gp160 and its cleavage product gp120 were detected (Fig. 3, lane 1).

To determine the time course of gp160 binding by calreticulin, pulse-chase experiments were performed. BS-C-1 cells expressing gp160 were labeled with [35S]methionine for 5 min and chased for times between 0 and 120 min. Immunoprecipitation of extracts from these cells by calreticulin-directed antibodies followed by SDS-PAGE showed that newly synthesized gp160 coprecipitated with calreticulin (Fig. 4A). Quantitation of the radioactivity in the bands revealed that the highest amount of gp160 was bound to calreticulin about 10 min after synthesis (Fig. 4B). After a chase of 25 min, the labeled gp160 bound to calreticulin was reduced by 50%, and by 90 min only trace amounts of gp160 were still associated with calreticulin. In the extracts of cells lysed directly after the 5 min pulse, the amounts of gp160 precipitated by either the calreticulin- or gp160-directed antibodies were less than that after a chase of at least 5 min. The delay could reflect continued incorporation of labeled methionine as well as the time needed for glycosylation (18). Because vaccinia virus infection stops host cell pro-
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protein synthesis (19), metabolically labeled calreticulin was not detected in infected cells (data not shown). Nevertheless, in Western blot of those cells (Fig. 3) or in radioimmunoprecipitation assays of uninfected cells (Fig. 1), calreticulin was easily detectable.

Calreticulin Binds to the N-terminal 204 Amino Acid Residues of HIV-1 gp160—The failure of calreticulin to bind gp120 could result either from the lack of binding sites in the N-terminal portion of gp160 or because cleavage of gp160 to form gp120 occurs after exit from the ER. To differentiate between these possibilities, we infected cells with recombinant vaccinia viruses expressing truncated HIV-1 gp160 proteins (15). Extracts of infected cells pulse-labeled with \[^{35}\text{S}\]methionine and chased for only 10 min were immunoprecipitated with anti-gp160 or anti-calreticulin serum. Coprecipitation with anti-calreticulin was detected even for the smallest protein comprising only the N-terminal 204 amino acids, less than half of that of gp120 (Fig. 5, right panel, lane 7). Similar results were obtained using a set of HIV type 2 gp160 truncation variants (16). In this case, calreticulin still bound to the N-terminal 198 amino acids (data not shown). Therefore, the failure of calreticulin to bind naturally cleaved gp120 must be due to its formation beyond the ER compartment.

Calreticulin Does Not Form Stable Complexes with Nonglycosylated gp160—Produced in the Presence of Tunicamycin—Calnexin binds to the N-linked oligosaccharides of newly synthesized proteins. Tunicamycin, which blocks all N-linked glycosylation, and castanospermine and 1-deoxynojirimycin, which both inhibit glucosidases I and II, were found to hinder the binding of calnexin to VSV G protein or influenza virus hemagglutinin, whereas 1-deoxymannojirimycin, which inhibits the ER α-mannosidase, did not interfere (20–22). It was concluded that calnexin is specific for very early folding intermediates that possess monoglucosylated core glycans (22). To determine if calreticulin would show such a lectin-like binding, we expressed gp160 in the presence of the inhibitors mentioned above as well as bromoconduritol, which inhibits only glucosidase II. Extracts of \[^{35}\text{S}\]methionine-labeled cells were immunoprecipitated by calreticulin-, calnexin-, or gp160-directed antibodies. The mobility differences displayed by the gp160 bands in inhibitor-free controls (Fig. 6, lane –) and castanospermine-, 1-deoxynojirimycin-, bromoconduritol-, and 1-deoxymannojirimycin-treated cells (Fig. 6, lanes CST, dNM, Br, and dMAN, respectively), showed that these compounds inhibited the trimming of N-linked sugars. In the presence of tunicamycin, the HIV-1 protein had increased mobility due to the absence of N-glycosylation. Only tunicamycin totally blocked the coprecipitation of gp160 with calnexin or calreticulin. Although castanospermine had no effect on the amount of gp160 precipitated by anti-gp160 serum, the amounts co-precipitated with calreticulin and calnexin were 15 and 26% of that co-precipitated in the absence of the drug. The other processing blockers had little or no effect (Fig. 6). The differences in the effects caused by castanospermine and 1-deoxymannojirimycin were not surprising even though both compounds affect the same enzymes. Hammond et al. (20) observed that 1-deoxymannojirimycin blocked binding of calnexin to newly synthesized proteins less efficiently than castanospermine, suggesting 1-deoxymannojirimycin is less efficient, as described previously (23).

Tunicamycin-treatment of uninfected BS-C-1 cells and immunoprecipitation with calreticulin-directed serum of their \[^{35}\text{S}\]methionine-labeled extracts led to similar results: tunicamycin prevented newly synthesized cellular proteins from binding to calreticulin (not shown).

gp160 Is Not Released from Calreticulin in the Presence of ATP—A specific property of many chaperones is their ability to bind selectively to unfolded polypeptides and dissociate from them through an ATP-hydrolizing reaction that is coupled to protein folding (24). According to recently published results, calreticulin binds to denatured proteins and can be released by the addition of ATP in the presence of divalent cations (11). We attempted to confirm this using two different approaches. First, gp160-calreticulin complexes were attached to protein A-Sepharose beads, washed three times in TNT buffer, and washed once in 150 mM NaCl, 1 mM dithiothreitol, 20 mM Tris, pH 8.0. The beads were then incubated in buffer containing 2 mM ATP, 2 mM Mg\(^{2+}\), 0.5 mM Ca\(^{2+}\) or 2 mM Mg\(^{2+}\), 0.5 mM Ca\(^{2+}\) or 2 mM ATP, 2 mM Mg\(^{2+}\), 0.5 mM Ca\(^{2+}\), 2.5 mM EDTA, 1 mM EGTA. In addition, one aliquot was incubated without divalent...
cations, ATP, EDTA, or EGTA. After 30 min at room temperature, the beads were washed and subjected to SDS treatment, PAGE, and autoradiography. The majority of gp160 remained bound to calreticulin despite addition of ATP (not shown). To determine whether the antibodies bound to calreticulin inhibited ATP-mediated release, we incubated the gp160-calreticulin complexes in cell extracts under the four different conditions described above prior to the addition of antibodies. Again, ATP did not induce dissociation of gp160 from calreticulin. Therefore, gp160 was not released from calreticulin by ATP in the presence of Mg$^{2+}$ and Ca$^{2+}$, either when the complex was in solution or bound to calreticulin-directed antibodies.

Comparison of the Binding Kinetics of Calnexin and Calreticulin to gp160—To compare the timing of gp160 binding to calreticulin and to calnexin, additional pulse-chase experiments were performed. BS-C-1 cells expressing gp160 were pulse-labeled with [$^{35}$S]methionine for 5 min and chased for times between 0 and 120 min. Half of the extracts from these cells were incubated with calreticulin-directed antibodies, and half of them were incubated with anti-calnexin antibodies followed by SDS-PAGE (Fig. 7A). Quantitation of the radioactivity in the bands revealed that calreticulin- and calnexin-directed antibodies bound the highest amounts of gp160 at about 5 and 10 min after synthesis, respectively (Fig. 7B). After a chase of 25 min, the number of gp160 molecules bound to calreticulin was reduced by 50%, and after 90 min only trace amounts of gp160 were still associated with calreticulin as described in a preceding section. For calnexin, we found the $t_{1/2}$ of dissociation to be 35 min. Thus, calreticulin and calnexin bind gp160 during the same period.

To find out whether calnexin and calreticulin bind sequentially or simultaneously to gp160, successive immunoprecipitations were performed. Again, BS-C-1 cells expressing gp160 were pulse-labeled with [$^{35}$S]methionine for 5 min and chased for times between 0 and 60 min. Half of each extract from these cells was depleted of calreticulin-bound gp160 by two successive immunoprecipitations with calreticulin-directed serum prebound to protein A-Sepharose; the other half of each extract was depleted of calnexin-bound gp160 by two successive immunoprecipitations with calnexin-directed serum. This procedure was followed by incubation of the supernatants with a surplus of protein A-Sepharose to remove remaining antibodies left from the first two rounds of immunoprecipitations. Finally, the samples depleted for calreticulin-bound gp160 still contained significant amounts of calnexin-bound gp160 (Fig. 8A). However, calnexin-directed serum effectively depleted calreticulin-bound gp160 because only trace amounts of gp160 were still precipitable by anti-calreticulin (Fig. 8A). This result suggests that under our conditions the majority of gp160 molecules that are bound to calreticulin are also bound to calnexin in a transient ternary complex, whereas there are many gp160-calnexin complexes without calreticulin. To rule out the trivial possibility that the calreticulin-gp160 complex was unstable or lost during the anti-calnexin steps, the immunoprecipitations were repeated with unrelated antibodies in the first two rounds (Fig. 8B). After two immunoprecipitations with VSV G protein-directed antibodies, there were still high amounts of calnexin-bound gp160 as well as calreticulin-bound gp160.
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Calreticulin is a highly conserved ER membrane protein that shares several regions of 42-78% identity with calnexin (25). The sequence similarity between calnexin and calreticulin suggests that these distinct ER proteins may have common functions. Calnexin associates transiently and selectively with newly synthesized glycoproteins, indicating that it may act as a chaperone (17, 20, 26, 27). Moreover, calnexin binds specifically to molecules with monoglucosylated core glycans, consistent with a role in the synthesis and secretion of glycoproteins (22). In addition to the structural similarities between calreticulin and calnexin, there have been several reports that calreticulin binds to a wide variety of cellular proteins and gp160 early during their processing and its specific binding to glycosylated proteins confirms this assumption. This role for calreticulin may also explain a recent finding that long term sensitization training in aplysia lead to an increase of the expression of calreticulin (30). Because similar findings were published by the same group for BiP (31), a chaperone function of calreticulin and BiP might serve to fold proteins necessary for the structural changes characteristic of long term memory.

Because of the striking structural and functional similarities between calreticulin and calnexin, we compared the kinetics of gp160 binding to calreticulin with its binding to calnexin and observed similar patterns. About 10 min after synthesis, a maximal amount of gp160 was bound to calnexin and calreticulin and about half remained bound after 25-35 min. The long t1/2 of dissociation is consistent with earlier reports that gp160 is processed rather slowly (18). Ou and co-workers (17) showed recently that maximal binding of a1-antitrypsin, complement C3, transferrin, and apolipoprotein B-100 to calnexin occurred 2-10 min after the pulse. This is probably due to the time needed for the translation of nascent polypeptide chains. Re-
cently, it was shown that association of calnexin to newly synthesized hemagglutinin starts when the chains are about half translated (32). The $t_{1/2}$ of dissociation of the proteins investigated by Ou et al. (17) from calnexin varied between 5 and 35 min.

Calnexin coprecipitated gp160 and small amounts of calreticulin (data not shown). This could be explained by the existence of either a ternary complex composed of gp160, calnexin, and calreticulin or of two binary complexes in which calnexin is bound to calreticulin or to gp160. However, the very similar binding kinetics of both chaperones to gp160 suggested the existence of a ternary complex. To prove that both chaperones bind to the same gp160-folding intermediates, extracts of cells were depleted of gp160 bound to calnexin by anti-calnexin antibodies, and the supernatants were assayed for calreticulin-bound gp160 and vice versa. Calnexin-directed serum depleted calreticulin-bound gp160 and vice versa. Calnexin-directed serum depleted calreticulin-bound gp160, suggesting the existence of a transient ternary complex composed of gp160, calnexin, and calreticulin. However, calreticulin-directed serum did not efficiently deplete calnexin-bound gp160. We ruled out the possibility that this effect results from differences in the stability of complexes between gp160 and calnexin or calreticulin. We concluded that calreticulin is mostly bound to gp160 molecules that are also bound to calnexin, whereas calnexin-gp160 complexes lacking calreticulin exist. Whether calnexin is required for calreticulin binding to gp160 cannot be deduced from our data. However, because calnexin is a transmembrane protein, whereas calreticulin is luminal, we consider a model in which calnexin holds the newly translated gp160 in the ER, whereas calreticulin associates with and dissociates from the protein. Alternatively, calnexin may fulfill its role on the parts of nascent proteins associated or close to the ER membrane, whereas the soluble calreticulin may manipulate the luminal parts of those proteins.

Acknowledgments—We thank Jonathan W. Yewdell and Patricia L. Earl for helpful discussions and Norman Cooper for providing cells and viruses.

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doi: 10.1074/jbc.271.1.97

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