Assembly of the multisubunit T cell antigen receptor (TCR) complex is an intricate process requiring coordinated regulation of at least six different gene products (α, β, γ, δ, ε, and ζ) and the ordered pairing of partner chains within the endoplasmic reticulum (ER). To date, two proteins have been implicated as functioning as molecular chaperones in the assembly of nascent TCR proteins: calnexin, a resident ER transmembrane protein, which associates with all TCR components except ζ and T cell receptor-associated protein, which selectively associates with CD3εα pairs. In this study, we examined the association of calreticulin, a soluble protein with significant sequence homology to calnexin, with newly synthesized TCR proteins. Analogous to calnexin, processing of glycan chains by glucosidase enzymes was required for initial association of TCRα and -β proteins with calreticulin; however, several major differences were noted regarding interaction of calnexin and calreticulin chaperones with TCR proteins. First, TCRα and -β proteins showed prolonged association with calnexin molecules compared with calreticulin; interaction of TCRα proteins with calreticulin was particularly transient, with most calreticulin-TCRα protein complexes dissociating within 15 min of their initial assembly. Second, we found that, unlike calnexin, which associated with clonotypic TCRα and -β proteins and invariant CD3δεα pairs, calreticulin associated specifically with clonotypic TCRα and -β proteins. These studies identify calreticulin as a molecular chaperone for nascent clonotypic TCRα and -β proteins and demonstrate that calreticulin and calnexin differentially associate with newly synthesized TCR proteins within the ER.

Assembly of complete αβδεγζ ζ T cell antigen receptor (TCR) complexes occurs within the endoplasmic reticulum (ER) and proceeds in a precisely ordered fashion (1, 2). Oligomerization of TCR proteins into TCR complexes is believed to be facilitated by their association with the molecular chaperone calnexin (3–5), a resident ER transmembrane protein that participates in the assembly of other multisubunit immune recep-

† To whom correspondence should be addressed: Dept. of Microbiology and Immunology, East Carolina University, School of Medicine, Greenville, NC 27858. Tel.: 919-816-2700; Fax: 919-816-3104.

‡ To whom correspondence should be addressed: Dept. of Microbiology and Immunology, East Carolina University, School of Medicine, Greenville, NC 27858. Tel.: 919-816-2700; Fax: 919-816-3104.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The related molecular chaperones calnexin and calreticulin differentially associate with nascent T cell antigen receptor proteins within the endoplasmic reticulum.

Jeroen E. M. Van Leeuwen, and Kelly P. Kearse‡
From the Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20892-1360

The abbreviations used are: TCR, T cell antigen receptor; ER, endoplasmic reticulum; cas, castanospermine; mAb, monoclonal antibody; PAG, polyacrylamide gel electrophoresis; NEM, non-equilibrium pH gradient electrophoresis.

Experimental Procedures

Assembly of TCR complexes, including major histocompatibility class I molecules (4, 6, 7), class II molecules (8), and the B cell antigen receptor (4, 9). Efficient association of TCR glycoproteins with calnexin requires removal of two Glc residues from nascent immature Glc3Man9(GlcNAc)2 glycan chains (5), a process referred to as Glc trimming (5, 10, 11). Glc trimming occurs in the ER by the sequential action of glucosidase I and II enzymes, which remove the outermost and two innermost Glc residues, respectively (12, 13). Because calnexin specifically recognizes monoglucosylated (Glc3Man9(GlcNAc)2) oligosaccharide species, both glucosidase I and II activities are necessary for creation of glycan substrates for calnexin binding (10, 14). Indeed, association of nascent glycoproteins with calnexin is significantly impaired by drug-induced blockade of glucosidase activity and in mutant cell lines that are deficient in expression of glucosidase enzymes (5, 12). Oligosaccharide chains are not strictly required for calnexin association, however, as several nonglycosylated molecules associate stably with calnexin, including recombinant multidrug resistance P glycoprotein (15) and CD3εα proteins (16).

Four individual TCR proteins have been demonstrated to interact with calnexin: clonotypic TCRα and TCRβ polypeptides (3, 5) and invariant CD3δ and CD3εα chains (3, 16, 17). Association of calnexin with CD3εα and CD3δεα pairs has also been observed, which, interestingly, are localized on the cell surfaces of immature T cells (18, 19). Assembly of calnexin with ζ proteins has never been reported (3). In the current study we examined the association of calreticulin with newly synthesized TCR proteins (20, 21). We found that the interaction of TCRα and -β proteins with calnexin and calreticulin chaperones was quite distinct in that TCRα and -β proteins associated much more transiently with calreticulin molecules than with calnexin. Moreover, in contrast to calnexin, which associated with both clonotypic TCRα and -β proteins and invariant CD3δεα and -ε chains, we found that calreticulin assembly was restricted to clonotypic, antigen-reactive TCRα and -β chains.

Metabolic Labeling, Cell Lysis, Immunoprecipitation, and Gel Electrophoresis—Metabolic pulse labeling was performed as described previously (2). Cells were solubilized in 1% Nonidet P-40 (Calbiochem) lysis buffer (20 mM Tris, 300 mM NaCl, plus protease inhibitors) or 1%
Calnexin and Calreticulin Association with T Cell Receptor Proteins

RESULTS

Initially, BW thymoma cells were metabolically labeled with \( ^{35}S \)-methionine for 30 min and solubilized in 1% digitonin, and lysates were precipitated with anti-calnexin Ab or anti-calreticulin Ab; precipitates were analyzed on two-dimensional NEPHGE-SDS-PAGE gels. Numerous proteins were associated with calnexin and calreticulin molecules in BW lysates, including proteins that comigrated with clonotypic TCR\( \alpha \) and \(-\beta\) polypeptides (Fig. 1A, top). Interestingly, when compared with anti-calnexin precipitates, the ratio of TCR\( \alpha \)-TCR\( \beta \) proteins coprecipitating with calreticulin was markedly skewed toward TCR\( \beta \) proteins (Fig. 1A, top). As shown in experiments using the glucosidase inhibitor cas (13), assembly of nascent TCR\( \alpha \) and -\( \beta \) glycoproteins with both calnexin and calreticulin chaperones was dependent on processing of glycan chains by ER glucosidase enzymes (Fig. 1A, bottom). These results were confirmed in the glucosidase II-deficient BW variant BWPHAR2.7 (22), in which calreticulin assembly with TCR\( \alpha \) and -\( \beta \) glycoproteins, as well as numerous unidentified proteins, was severely decreased relative to parental BW cells (Fig. 1B). Finally, as determined by immunoprecipitation and immunoblotting experiments, the anti-calnexin and anti-calreticulin Abs used in these studies were not cross-reactive (Fig. 1C), indicating that TCR\( \alpha \) and -\( \beta \) proteins were specifically associated with calnexin and calreticulin molecules in BW lysates. Thus, we conclude that newly synthesized TCR\( \alpha \) and -\( \beta \) proteins associate with both calnexin and calreticulin, and that these interactions require processing of glycan chains by ER glucosidase enzymes.

In our next set of studies, the stability of calnexin-TCR\( \alpha \) and -\( \beta \) and calreticulin-TCR\( \alpha \) and -\( \beta \) interactions was evaluated. To specifically examine association of TCR\( \alpha \) proteins with calnexin and calreticulin chaperones, BW cells were pulse labeled with \( ^{35}S \)-methionine for 5 min and chased for various periods in medium containing excess nonradioactive methionine. Digitonin lysates were precipitated with anti-calnexin and anti-calreticulin Abs; precipitates were boiled in 1% SDS to release bound material, Nonidet P-40 detergent was added to counteract the SDS, and TCR\( \alpha \) proteins were recaptured by precipitation with anti-TCR\( \alpha \) mAb (2). As demonstrated, the vast majority of TCR\( \alpha \)-calnexin complexes formed during a short 5-min pulse were stable during a 30-min chase period (Fig. 2). In contrast, most nascent TCR\( \alpha \) proteins associated with calreticulin.
Recapture Ab: Anti-TCRα

Chase (min): 0 5 10 20 30

Immpt. Ab: Anti-Calnexin

TCRα-

Recapture Ab: Anti-Calreticulin

Chase (min): 0 5 10 20

Immpt. Ab: Anti-Calnexin

Antic-Calreticulin

Fig. 3. Deglucosylation is an important step in the dissociation of TCRα proteins from calreticulin. BW cells were metabolically pulse labeled for 5 min with [35S]methionine (in the absence of cas) and chased in the presence (MED) or absence (CAS) of cas for the periods indicated. Cells were solubilized in 1% digitonin, and lysates were immunoprecipitated (Immpt.) with anti-calreticulin (Anti-Crt) Abs; bound material was released by boiling in 1% SDS, and TCRα proteins were recaptured as in Fig. 2. The position of TCRα proteins is indicated.

Fig. 4. Stability of calreticulin-TCRα and calreticulin-TCRβ protein complexes. BW cells were metabolically pulse labeled for 5 min with [35S]methionine and chased for the periods indicated. Cells were solubilized in 1% digitonin, and lysates were immunoprecipitated (Immpt.) with anti-calreticulin Ab; precipitates were analyzed directly on two-dimensional NEPHGE/SDS-PAGE under reducing conditions. The positions of calreticulin (arrow) and TCRα and TCRβ proteins are indicated.

In the current report we examined the association of newly synthesized TCR proteins with calreticulin. These studies demonstrate that, analogous to calnexin, glycan processing is required for assembly of calreticulin with TCRα and -β proteins in the ER, that nascent TCRα and -β proteins are more stably associated with calnexin than with calreticulin, and finally, released by boiling in SDS, and CD3 proteins were recaptured with various anti-CD3-specific Abs. As demonstrated, CD3δ and CD3ε proteins were recaptured from anti-calnexin precipitates, but not from anti-calreticulin precipitates, of BWδ and 2B4 T cell lysates (Fig. 6). Identical results were observed in splenic T cells; and association of CD3δ and -ε components with calnexin but not calreticulin in T cells was confirmed by immunoblotting (data not shown). Therefore, we conclude that calreticulin associates specifically with nascent, clonotypic TCRα and -β proteins but not with newly synthesized, invariant CD3δ and -ε chains.

DISCUSSION

In the current report we examined the association of newly synthesized TCR proteins with calreticulin. These studies demonstrate that, analogous to calnexin, glycan processing is required for assembly of calreticulin with TCRα and -β proteins in the ER, that nascent TCRα and -β proteins are more stably associated with calnexin than with calreticulin, and finally,
that calreticulin associates specifically with clonotypic TCRα and -β proteins but not with invariant CD3δ and -ε chains.

Similar to what has been observed with calnexin (5), we found that Glc trimming was necessary for efficient association of newly synthesized TCRα and -β proteins with calreticulin. Thus, as recently proposed by several laboratories (11, 12, 29–32), calreticulin represents the second member of a family of endogenous lectin molecules specific for glycoproteins containing partially glycosylated glycans, with calnexin being the first (10). Despite intensive investigation over the past few years, the precise function of calnexin in the assembly of nascent TCR complexes within the ER remains to be delineated. Current hypotheses suggest that calnexin interacts with individual newly synthesized TCR proteins to facilitate their folding within the ER, prevent their escape to the Golgi compartment, and catalyze their oligomerization into complete αβδεγεζζ TCR complexes (3–5, 16, 17). The identification of both calnexin and calreticulin as chaperones for TCRα and -β glycoproteins containing incompletely trimmed glycan chains raises the intriguing possibility that calnexin and calreticulin perform distinct molecular functions in the folding and assembly of nascent TCRα and -β chains within the ER. It is noteworthy to mention that monoglycosylated (Glc₅Man₉GlcNAc₂) glycans can be generated in the ER by two different mechanisms: either directly via the Glc-trimming pathway or indirectly via reglucosylation of processed Man₆GlcNAc₂ species by ER glucosyltransferase enzymes (11, 14, 33, 34). Thus, it is interesting to speculate that calreticulin and calnexin may differentially associate with monoglycosylated glycoproteins derived via the Glc-trimming and Glc-trimming and reglucosylation pathways in the ER. Alternatively, calnexin and calreticulin chaperones may function redundantly in the ER quality control system to maximize the probability that nascent TCRα and -β chains (and other neoglycoproteins) are correctly folded. Regarding this issue, recent studies by Scott and Dawson (35) have demonstrated that major histocompatibility complex class I proteins are efficiently assembled and expressed in a human cell line deficient in calnexin expression (35), indicating that “backup” mechanisms exist within the ER quality control system for correct folding and assembly of oligomeric protein complexes.

In contrast to calnexin association with TCRα and -β proteins and calreticulin association with TCRβ proteins, the association of calreticulin with nascent TCRα proteins was remarkably unstable, with most TCRα proteins rapidly dissociating from calreticulin molecules within 15 min of their assembly. Blockade of glucosidase activity by cas treatment stabilized calreticulin-TCRα protein interactions, indicating that removal of Glc residues from nascent glycan chains is an important step in the disassembly of TCRα proteins from calreticulin. In contrast, calnexin-TCRα protein complexes were not stabilized by cas treatment, providing another interesting difference between calnexin and calreticulin and their function as molecular chaperones for nascent TCR proteins.

Finally, the current report shows that, in contrast to calnexin, which associates with both clonotypic TCRα and -β proteins and invariant CD3δ and -ε proteins, calreticulin association is restricted to clonotypic, antigen-reactive chains. Although these studies do not formally exclude the possibility that weak interactions between invariant CD3δ and -ε chains and calreticulin molecules may exist, our data clearly show that protein complexes of calnexin-CD3δ and -ε chains and calreticulin-TCRα and -β chains were stably isolated under conditions in which association of calreticulin with CD3 chains was not detected. It is conceivable that, unlike the extracellular regions of TCRα and -β molecules, the extracellular domains of CD3δ and -ε chains do not sufficiently extend into the ER lumen, thereby precluding their (stable) association with soluble calreticulin molecules (1). Alternatively, it is possible that invariant CD3δ and -ε chains lack the structural domains necessary for their assembly with calreticulin molecules. Elucidation of the structural determinants on TCR proteins important for their interaction with calnexin and calreticulin molecules should provide valuable information regarding these issues.

Acknowledgments—We thank Drs. Juan Bonifacino, Randy Ribaudo, David Segal, Alfred Singer, and David Wiest for critical evaluation of the manuscript; Drs. Juan Bonifacino, Randy Ribaudo, and Larry Samelson for gifts of antibodies; and Drs. Ken Katz and Alfred Singer for providing BW5 cells.

REFERENCES

1. Klausner, R. D., Lippincott-Schwartz, J., and Bonifacino, J. S. (1990) Annu. Rev. Cell Biol. 6, 403–432.
2. Kearse, K. P., Roberts, J. L., and Singer, A. (1995) Immunity 2, 391–399.
3. Melnick, J., and Argon, Y. (1995) Immunol. Today 16, 243–250.
4. Hochstenbach, F., David, V., Watkins, S., and Brenner, M. B. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4734–4738.
5. Kipnis, J., and Singer, A. (1995) Cell 80, 211–221.

FIG. 5. Prolonged association of TCRα and -β proteins with calnexin but not calreticulin. BW cells were metabolically pulse labeled for 5 min with [³⁵S]methionine and chased for the periods indicated. Cells were solubilized in 1% digitonin, lysates were immunoprecipitated with anti-calnexin or anti-calreticulin Ab, and precipitates were analyzed on two-dimensional NEPHGE/SDS-PAGE under reducing conditions. The positions of TCRα and TCRβ proteins are indicated.

FIG. 6. Differential assembly of newly synthesized, invariant CD3δ and -ε proteins with calnexin and calreticulin. Digitonin lysates of radiolabeled BW5 cells and 2B4 T hybridoma cells were immunoprecipitated with anti-calnexin (Anti-Cx) or anti-calreticulin (Anti-Crt) Abs, precipitates were boiled in 1% SDS to release bound material, Nonidet P-40 detergent was added, and TCR proteins were recaptured with anti-CD3δ (R9) antiserum or anti-CD3δ mAb (HMT3.1). The positions of CD3δ and -ε proteins are marked. *, unknown protein that is specifically recaptured with anti-CD3ε mAb; this protein is not CD3δ, as it is not glycosylated (data not shown). Note that radiolabeled CD3δ and -ε proteins were not visible in recaptures from anti-calreticulin precipitates, even on prolonged exposure of autoradiographs.
Calnexin and Calreticulin Association with T Cell Receptor Proteins

5. Kearse, K. P., Williams, D. B., and Singer, A. (1994) EMBO J. 13, 3678–3686
6. Degen, E., and Williams, D. B. (1991) J. Cell Biol. 112, 1099–1115
7. Belov, J. P., Weissman, J. D., and Kearse, K. P. (1995) J. Biol. Chem. 270, 29025–29029
8. Anderson, K. S., and Cresswell, P. (1994) EMBO J. 13, 675–682
9. Grupp, S. A., Mitchell, R. N., Schreiber, R. L., McKenzie, D. J., and Abbas, A. K. (1995) J. Exp. Med. 181, 161–168
10. Ware, F. E., Vassilakos, A., Peterson, P. A., Jackson, M. R., Lehrman, M. A., and Williams, D. B. (1995) J. Biol. Chem. 270, 29025–29029
11. Hebert, D. N., Foellmer, B., and Helenius, A. (1995) Cell 81, 425–433
12. Birke, A. B. (1991) FASEB J. 5, 3055–3063
13. Hammond, C., Braakman, I., and Helenius, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 913–917
14. Lou, T. W., and Clarke, D. M. (1994) J. Biol. Chem. 269, 26683–26689
15. Rajagopal, S., Xu, Y., and Brenner, M. B. (1994) Science 263, 387–390
16. David, V., Hochstenbach, B., Rajagopal, S., and Brenner, M. B. (1993) J. Biol. Chem. 268, 9585–9592
17. Wiest, D. L., Kearse, K. P., Shores, E. W., and Singer, A. (1994) J. Exp. Med. 180, 1375–1382
18. Michalak, M., Milner, R. E., Burns, K., and Opas, M. (1992) Biochem. J. 285, 681–692
19. Woda, I., Rindress, D., Cameron, P. H., Ou, W. J., Doeherty, J. J., Leuvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. M. (1991) J. Biol. Chem. 266, 19599–19610
20. Woda, I., Rindress, D., Cameron, P. H., Ou, W. J., Doeherty, J. J., Leuvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. M. (1991) J. Biol. Chem. 266, 19599–19610
21. Hyman, R., and Stallings, V. (1974) J. Natl. Cancer Inst. 52, 429–437
22. Hedrick, S. M., Matis, L. A., Hecht, T. T., Samelson, L. E., Longo, D. L., Heber-Katz, E., and Schwartz, B. H. (1982) Cell 30, 141–152
23. Leu, O., Foo, M., Sachs, D. H., Samelson, L. E., and Bluestone, J. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1374–1378
24. Born, W., Miles, C., White, J., O’Brien, R., Freed, J. H., Marrack, P., Kappler, J., and Kubo, R. T. (1987) Nature 330, 572–574
25. Becker, J. B., Near, R., Mudgett-Hunter, M., Margolis, M. M., Kubo, R. T., Kaye, J., and Hedrick, S. M. (1989) Cell 58, 911–921
26. Kubo, R. T., Born, J. W., Kappler, J., Marrack, P., and Pigeon, M. (1989) J. Immunol. 142, 2736–2742
27. Samelson, L. E., Weissman, A. M., Robey, F. A., Berkower, L., and Klausner, R. D. (1986) J. Immunol. 137, 3254–3258
28. Nauseef, W. M., McCormick, S. J., and Clark, R. A. (1995) J. Biol. Chem. 270, 4741–4747
29. Wada, I., Imai, S. I., Kai, M., Sakane, F., and Kanoh, H. (1995) J. Biol. Chem. 270, 20286–20304
30. Powell, L., and Varki, A. (1995) J. Biol. Chem. 270, 13423–13426
31. Peterson, J. R., Ori, A., Van, P. N., and Helenius, A. (1995) Mol. Biol. Cell 6, 1173–1184
32. Sousa, S., and Parodi, A. (1995) EMBO J. 14, 4196–4203
33. Parodi, A. J., Mendelzon, D. H., and Lederkremer, G. Z. (1983) J. Biol. Chem. 258, 8260–8265
34. Scott, J. E., and Dawson, J. R. (1995) J. Immunol. 222, 143–148