

# Regulating the Retention of T-Cell Receptor $\alpha$ Chain Variants within the Endoplasmic Reticulum: $\text{Ca}^{2+}$ -dependent Association with BiP

Carolyn K. Suzuki, Juan S. Bonifacino, Augustin Y. Lin,\* Mark M. Davis,\* and Richard D. Klausner

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; and \*Department of Microbiology and Immunology and Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California 94305

**Abstract.** Immunoglobulin heavy chain binding protein (BiP, GRP 78) coprecipitates with soluble and membrane-associated variants of the T-cell antigen receptor  $\alpha$  chain (TCR- $\alpha$ ) which are stably retained within the ER. Chelation of  $\text{Ca}^{2+}$  during solubilization of cells leads to the dissociation of BiP from the TCR- $\alpha$  variants, which is dependent upon the availability of  $\text{Mg}^{2+}$  and hydrolyzable ATP; this suggests that  $\text{Ca}^{2+}$  levels can serve to modulate the association/dissociation of these proteins with BiP. In vivo treatment of cells expressing either the soluble or membrane-anchored TCR- $\alpha$  variants with the  $\text{Ca}^{2+}$

ionophore, A23187, or an inhibitor of an ER  $\text{Ca}^{2+}$ -ATPase, thapsigargin, or the membrane-permeant  $\text{Ca}^{2+}$  chelator BAPTA-AM, results in the redistribution of these proteins out of the ER and their subsequent secretion or cell surface expression. Under the same assay conditions, no movement of BiP out of the ER is observed. Taken together, these observations indicate that decreased  $\text{Ca}^{2+}$  levels result in the dissociation of a protein bound to BiP, leading to its release from ER retention. These data suggest that the intracellular fate of newly synthesized proteins stably associated with BiP can be regulated by  $\text{Ca}^{2+}$  levels in the ER.

**T**HE insertion into or across the membrane of the ER is only the first step in a long pathway leading to the secretion of soluble proteins or the delivery of membrane proteins to the cell surface. Whether or not a protein will reach its intended destination depends upon its structural maturation within the ER. The process of structural maturation involves the folding of monomeric proteins as well as the assembly of oligomeric complexes (Hurtley and Helenius, 1989; Rose and Doms, 1988). Incomplete maturation often results in the failure to be transported through the secretory system, whereby immature proteins are either stably retained in the ER (Copeland et al., 1986; Kozutsumi et al., 1988; Hurtley et al., 1989) or are targeted for ER degradation (Lippincott-Schwartz, et al., 1988; Chen et al., 1988; Bonifacino et al., 1989).

Retention is most likely accomplished by the interaction of proteins with resident components of the ER. Currently, the best candidate for such a retention molecule is the Ig heavy chain binding protein, BiP (Haas and Wabl, 1983). For BiP to serve successfully as a retention molecule for immature or abnormal proteins it must be capable of recognizing either a determinant present in a wide variety of proteins, or, a variety of structural determinants. Just what structural features determine binding to BiP is unknown. Although it has been suggested that BiP might recognize exposed hydrophobic faces of denatured proteins (Pelham, 1986), recent evidence has shown that a variety of short hydrophilic peptides can bind to BiP with a wide range of affinities (Flynn et al., 1989).

The presence of a retention molecule in such great abundance as BiP, presents a dilemma regarding the maturation of newly synthesized proteins. All newly synthesized proteins are structurally immature and, as such, could be expected to interact with retention molecules. If this binding were too stable, these proteins would be prevented from folding or assembling and remain trapped within the ER. Indeed, the transient interaction of some newly synthesized proteins with BiP has been documented (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987; Ng et al., 1989; Machamer et al., 1990; Hendershot, 1990). Thus, as important as it is to understand how proteins associate with BiP, we must also understand how proteins are released from it. The first insight into resolving this problem came with the recognition that BiP is an ATP-binding protein (Lewis and Pelham, 1985; Chappell et al., 1986), and is, moreover, an ATPase; hydrolysis of ATP bound to BiP can result in the release of associated protein or peptide (Munro and Pelham, 1986; Kassenbrock and Kelly, 1989; Flynn et al., 1989). Thus, it is likely that the interaction with retention molecules in the ER is a dynamic process of binding and release. When a protein is released it can progress in its maturation, and this changes the probability of rebinding to abundant retention molecules. One can envision that this cycle repeats itself until the protein attains a configuration in which determinants that interact with BiP are either lost or masked.

There is much that we do not know about the association/dissociation cycle of a protein with BiP. Issues concerning the physiological role of ATP in this process and whether

there are special properties of the ER environment that might influence these interactions remains to be established. In this study, we have utilized variants of the T-cell antigen receptor  $\alpha$  chain (TCR- $\alpha$ ),<sup>1</sup> which are retained within the ER and tightly bound to BiP in order to explore the relationship between intracellular fate and BiP association/dissociation. In this report, two fundamental questions have been addressed: (a) is the association with BiP responsible for selectively trapping the TCR- $\alpha$  variants within the ER? and (b) can this interaction be modulated in vivo such that ER retention is converted to secretion or surface expression? Our findings have allowed us to identify intraluminal Ca<sup>2+</sup> as a factor involved in potentially regulating the binding and release of proteins from BiP within the ER, and, consequently, their fate within the secretory pathway. We provide evidence that the reduction of Ca<sup>2+</sup> levels in vitro as well as in vivo can influence the release of a proteins from BiP. In intact cells this manipulation is sufficient to cause the secretion or surface expression of proteins that are otherwise retained within the ER. This conversion from retention to secretion takes place in the absence of any breakdown of the ER or secretion of BiP. High levels of Ca<sup>2+</sup> in the ER may enhance the binding of newly synthesized proteins to BiP, whereas a drop in luminal Ca<sup>2+</sup> would provide a mechanism to enhance the release of such proteins. Thus, variance in Ca<sup>2+</sup> levels within the ER and associated compartments could allow for the spatial regulation of retention and release of proteins within this organelle.

## Materials and Methods

### Reagents

ATP and adenylyl-imidodiphosphate (AMP-PNP) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). A23187, apyrase, cycloheximide (CHX), EDTA, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride was supplied by Aldrich Chemical Co. (Milwaukee, WI). BAPTA-AM was purchased from Molecular Probes, Inc. (Eugene, OR). Thapsigargin was a kind gift from Dr. M.R. Hanley (MRC, Cambridge, England).

### Antibodies

The following antibodies were used for immunoprecipitation and/or immunofluorescence studies: the murine IgG2A mAb A2B4-2 (A2B4), recognizing a clonotypic determinant of the 2B4- $\alpha$  chain (Samelson et al., 1983); the rat anti-BiP (IgG $\kappa$ ) mAb (Bole et al., 1986) which was a most generous gift from Dr. D.G. Bole (University of Michigan, Ann Arbor, MI) and Dr. L.M. Hendershot (St. Jude's Children's Research Hospital, Memphis, TN); the murine mAb 7G7/B6 against an epitope localized to the extracellular domain of the IL-2 receptor  $\alpha$  chain (Rubin et al., 1985) provided by Dr. D. L. Nelson (National Institutes of Health, Bethesda, MD), and a polyclonal rabbit antiserum recognizing ER resident proteins (Louvard et al., 1982), kindly provided by Dr. D. Louvard (Pasteur Institute, Paris, France).

### Stably Transfected CHO Cells Expressing a Lipid-Linked Form of TCR- $\alpha$

Stable CHO transfectants expressing a lipid-linked form of the TCR- $\alpha$  subunit were produced as has been described previously by Lin et al. (1990). Briefly, the cDNA encoding the extracellular domain of TCR- $\alpha$  ending with the codon for Thr<sup>207</sup>, was fused in frame 5' to a 37 amino acid sequence of decay acceleration factor (DAF) which signals for the addition of a glycosylphosphatidylinositol anchor (TCR- $\alpha$ <sub>GPI</sub>). The extracellular

<sup>1</sup> Abbreviation used in this paper: TCR- $\alpha$ , T-cell antigen receptor  $\alpha$  chain.

domain of TCR- $\alpha$  in this membrane associated form is identical to that found in the truncated soluble form, TCR- $\alpha$ <sub>t</sub> (see below). Throughout this study, we have used a variant of this stably transfected cell line which expresses lower levels of TCR- $\alpha$ <sub>GPI</sub> as compared with that described by Lin et al. (1990); the steady state distribution of this protein localizes to the ER.

### Transient Expression of a Soluble Truncated TCR- $\alpha$ Chain in COS Cells

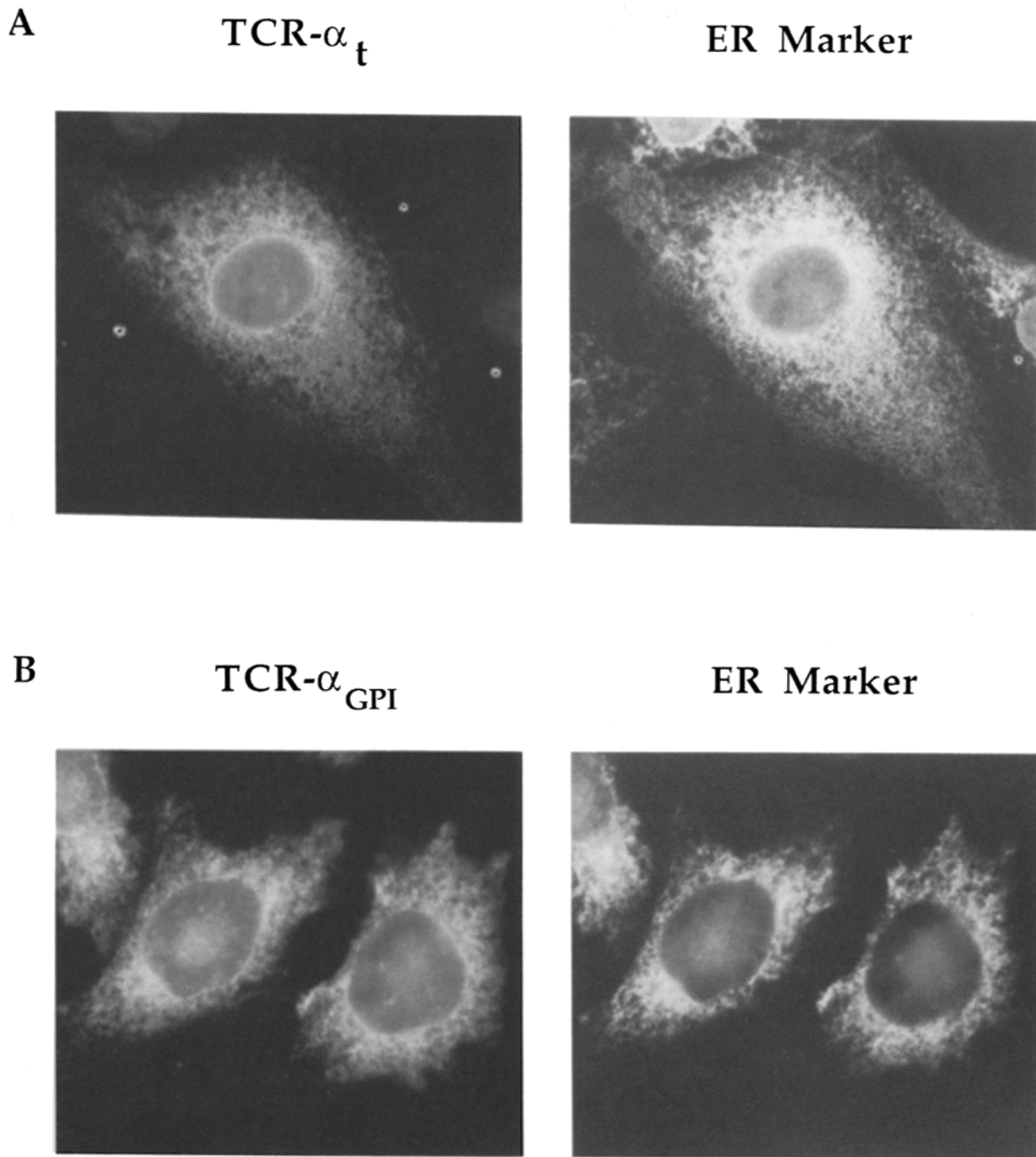
COS cells from the American Type Culture Collection (Rockville, MD) were transfected with the cDNA encoding a truncation of the clonotypic T-cell receptor 2B4- $\alpha$  chain as has been previously described (Bonifacino et al., 1990b). Briefly, a translation stop codon was introduced after the codon for Thr<sup>207</sup> in the extracellular domain of the mature 2B4- $\alpha$  protein (TCR- $\alpha$ <sub>t</sub>) by using M13-based mutagenesis following the procedure of Kunkel et al. (1987); the TCR- $\alpha$ <sub>t</sub> gene was cloned into an Xba I site present in a modified version of the expression vector pCDM8 (Seed, 1987). (In Figs. 2 and 6, COS cells were also transiently transfected with the expression vector pBJ1-neo encoding the cDNA for a lipid-linked form of TCR- $\alpha$  which has been previously described by Lin et al., 1990). COS fibroblast cells grown to 70–80% confluency in 150-mm-diameter tissue culture dishes were transfected with 1 ml calcium phosphate-precipitated DNA (20  $\mu$ g plasmid/ml), in 10 ml DME (Biofluids Inc., Rockville, MD) containing 10% FBS, 2 mM glutamine, and 0.15 mg/ml gentamicin (COS medium) as indicated previously by Bonifacino et al. (1989). After 16 h at 37°C, the medium was replaced by fresh COS medium and incubated for an additional 24 h.

### Metabolic Labeling, Immunoprecipitation, Electrophoresis, and Immunoblotting

36 h after transfection, COS cells were preincubated for 15 min at 37°C in methionine-free DME containing 5% FBS, 2 mM glutamine, and 0.15 mg/ml gentamicin. Cells were labeled for 30 min at 37°C with 0.25–0.5 mCi/ml [<sup>35</sup>S]methionine (Tran-<sup>35</sup>S-Label; ICN Radiochemicals, Irvine, CA) in methionine-free medium, and chased in COS medium for the times as indicated. Long-term labeling was performed using 1 mCi/ml [<sup>35</sup>S]methionine in medium that was nine parts methionine-free RPMI 1640 and one part methionine-containing RPMI 1640 (Biofluids Inc., Rockville, MD) supplemented with 10% FBS, 2 mM glutamine, and 0.15 mg/ml gentamicin. Cells and supernatants were collected separately. Cell pellets were solubilized in ice-cold lysis buffer containing 0.5% (wt/vol) Triton X-100, 0.3 M NaCl, 50 mM Tris-Cl, pH 7.4, which included protease inhibitors (Samelson et al., 1985). Insoluble material was removed by centrifugation for 15 min (or as indicated in figure legends), at maximum speed in a microfuge (Beckman Instruments Inc., Palo Alto, CA) at 4°C. Specific proteins were isolated from cell lysates or culture supernatants by immunoprecipitation using antibodies directed at the respective proteins coupled to protein A-agarose (Bethesda Research Laboratories, Bethesda, MD) as described (Samelson et al., 1985). Digestion with endo H was performed by incubating immunoprecipitated proteins for 16 h at 37°C with 2.5 mU endo H in 25  $\mu$ l buffer containing 0.1 M sodium phosphate buffer, pH 6.1, 0.1% Triton X-100, 0.03% SDS, and 20 mM EDTA. Samples were analyzed by one-dimensional SDS-PAGE according to Samelson et al. (1985) or by two-dimensional gel electrophoresis as described by O'Farrell et al. (1977).

### Immunofluorescence Microscopy

The distribution of TCR- $\alpha$  variants and BiP was determined morphologically in COS cells transiently expressing TCR- $\alpha$ <sub>t</sub>, or in CHO cells stably expressing a TCR- $\alpha$ <sub>GPI</sub>. Cells were grown to 50–60% confluency on 12-mm round coverslips and fixed with 3% formaldehyde in PBS for 10 min at room temperature. The cells were then permeabilized with 0.1% saponin in PBS for 15 min at room temperature before the addition of antibody-containing medium that contained 0.1% saponin (for these experiments either an undiluted A2B4 culture supernatant or a 1:20 dilution of anti-BiP culture supernatant or a 1:200 dilution of anti-ER antiserum was used). After incubation with the primary antibody, cells were washed in PBS with 0.1% BSA, and then incubated with the fluorescently labeled secondary antibody and washed again. The antibody incubations were each 1 h at temperature. Coverslips were mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL), and examined with an ICM405 inverted microscope (Carl Zeiss Inc., Thornwood, NY).



**Figure 1.** Immunofluorescent colocalization of TCR- $\alpha_t$  or TCR- $\alpha_{GPI}$  and ER resident proteins. (A) 36 h after transfection, COS cells transiently expressing TCR- $\alpha_t$  were fixed with formaldehyde, permeabilized with saponin, and stained with an antibody recognizing the TCR- $\alpha$  variant (A2B4) as well as an antiserum against ER proteins. (B) CHO cells stably expressing TCR- $\alpha_{GPI}$  were treated in the same fashion. The steady state distribution of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  yields a reticular pattern of immunofluorescence throughout the cytoplasm and distinct staining of the nuclear envelope which corresponds to that of the ER marker.

### ***Intracellular Ca<sup>2+</sup> Quantitation by Digital Imaging Microscopy***

Cells were labeled for 1.5–2 h at 37°C with a loading solution containing 4 mM Fura-2/AM, 0.07% DMSO, and 0.0145% F127 (a wetting solution added to insure uniform distribution of Fura-2); 2 mM probenecid was added to increase the efficiency of Fura-2/AM loading (Di Virgilio et al., 1990). Intracellular Ca<sup>2+</sup> levels were quantitated using a Gould FD5000 image processor (Fremount, CA) with software modified from Roger Y. Tsien (University of California at San Diego, CA) (Tsien and Harootunian,

1990); measurements were collected at 500 nm after alternate excitation at 350 and 380 nm.

### ***Results***

#### ***Variants of the T-Cell Receptor $\alpha$ Chain Are Stably Retained within the ER and Associated with BiP***

Over the past several years, we have utilized the multimeric

TCR to explore the relationship between the fates of newly synthesized proteins and their assembly into mature oligomeric complexes (Klausner et al., 1990). The TCR is a multi-subunit complex, which in most T cells is constituted by at least seven transmembrane proteins. The clonotypic  $\alpha$  and  $\beta$  subunits form a disulfide-linked heterodimer recognizing antigen in association with either class I or class II major histocompatibility molecules. The TCR- $\alpha\beta$  heterodimer is noncovalently associated with the invariant subunits CD3- $\delta$ ,  $-\gamma$ , and  $-\epsilon$ , complexed with the  $\zeta_2$  homodimer. The murine 2B4 TCR- $\alpha$  chain, which is used in the study reported here, is synthesized as an  $\sim M_r$  38,000 precursor that is posttranslationally modified into an  $M_r$  42,000–44,000 form in the Golgi system. We have recently shown that the full-length  $\alpha$  subunit of the receptor contains a determinant within its transmembrane domain that targets the protein for retention and rapid degradation in the ER. Interestingly, removal of the transmembrane and cytoplasmic domains of TCR- $\alpha$  results in a soluble, truncated variant, TCR- $\alpha_t$ , which is not secreted from the cell but retained within the ER (Bonifacino et al., 1990a,b). We wondered if the retention of this protein was a consequence of its disposition as a soluble protein within the ER lumen. We tested a membrane-associated form of TCR- $\alpha$  in which the extracellular domain, although identical to that in TCR- $\alpha_t$ , is linked to the ER membrane by a glycosylphosphatidylinositol anchor (TCR- $\alpha_{GPI}$ , Lin et al., 1990). As shown in Fig. 1, *A* and *B*, the steady state distribution of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$ , respectively, corresponded to the ER, as assessed by double-labeling immunofluorescence of the soluble- or lipid-linked variant and an ER marker. TCR- $\alpha_{GPI}$  stably expressed in CHO cells, like TCR- $\alpha_t$ , revealed a reticular pattern of fluorescence in addition to staining of the nuclear envelope coinciding with that of the ER marker.

Pulse-chase experiments revealed that the vast majority of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  was retained intracellularly as an endo H-sensitive form (Fig. 2), suggesting that neither had reached the medial Golgi cisternae, where carbohydrate modifications conferring endo H resistance occur (Tarentino and Maley, 1974). This is in marked contrast to the full-length TCR- $\alpha$  which is rapidly degraded within the ER with a half-life of 30–60 min (Bonifacino et al., 1989). Immunoprecipitation of the culture supernatant showed that 10–20% of TCR- $\alpha_t$  was released from the cells over 8 h as an endo H-resistant protein, with <1% of TCR- $\alpha_{GPI}$  shed into the medium.

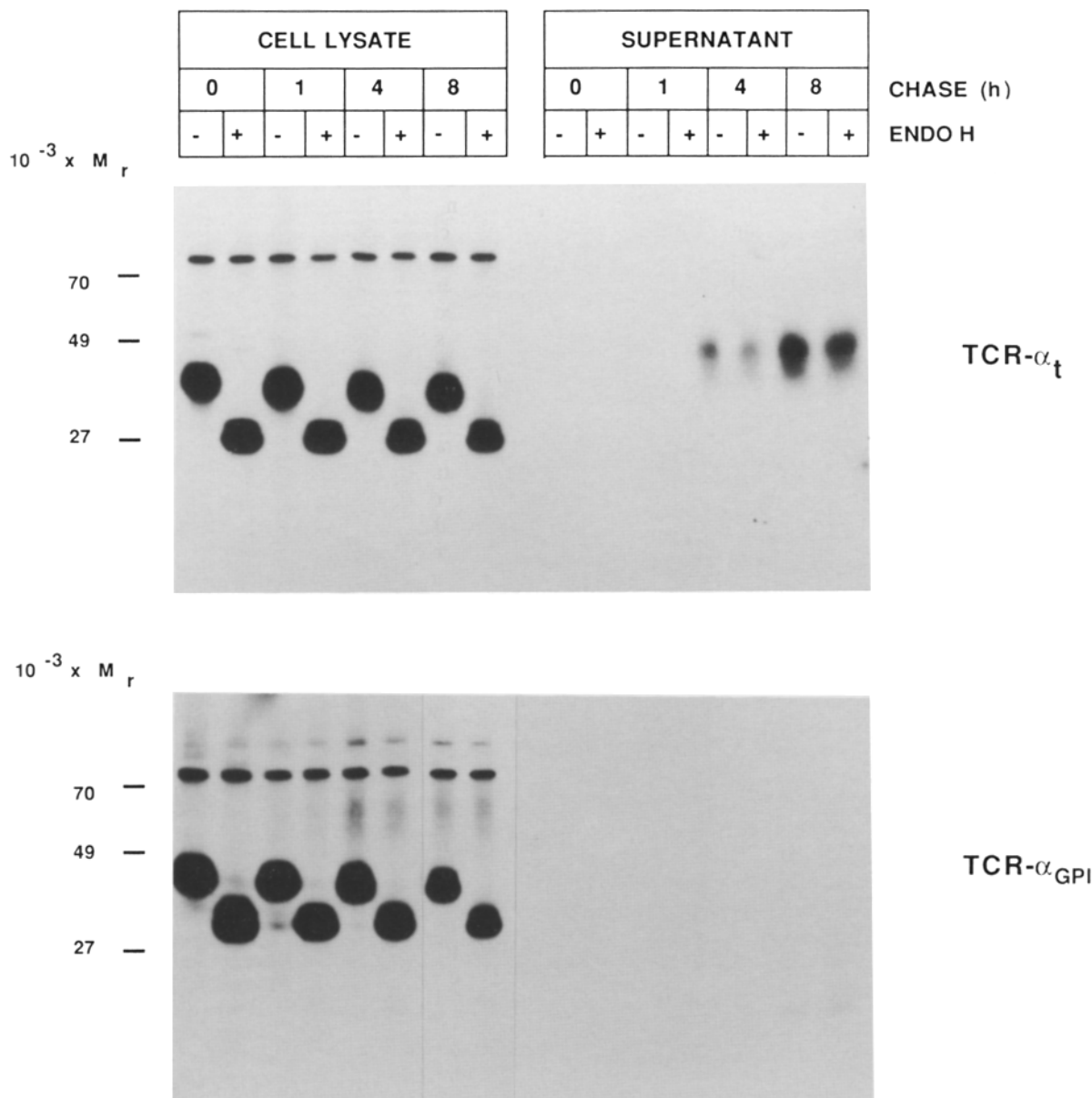
In view of our interest in understanding the ER retention of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$ , we were particularly struck by the coprecipitation of a 78-kD protein with the two TCR- $\alpha$  variants (Fig. 2). This protein was specifically coprecipitated with TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  isolated from cell lysates, but not with the fraction found in culture supernatants (Fig. 2). Identification of this protein as the immunoglobulin heavy chain binding protein, BiP, was accomplished by examining its migration on 2-D gels. Cells were labeled for 16 h with [ $^{35}$ S]methionine and lysates immunoprecipitated with mAbs recognizing TCR- $\alpha_t$ , which migrates at  $\sim 40$  kD (Fig. 3, *top panel*) and TCR- $\alpha_{GPI}$ , at  $\sim 43$  kD (Fig. 3, *middle panel*) or BiP (Fig. 3, *bottom panel*) (Bole et al., 1986). BiP (Fig. 3, *arrows*), migrated as an acidic protein to a pH of  $\sim 4$  in the first dimension of the nonequilibrium pH gradient elec-

trophoresis (NEPHGE), and with a molecular mass of 78 kD in the second dimension SDS-PAGE. Immunoprecipitation of COS or CHO lysates with an irrelevant antibody directed against the 2B4 TCR- $\beta$  chain, revealed that the 78-kD spot was specifically recognized by the anti-BiP antibody, whereas the minor spots migrating adjacent to BiP were nonspecific. Superimposition of autoradiograms and alignment of the origins and nonspecific spots showed that the overall migration of BiP coprecipitated with TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  was the same. In these experiments we did not observe coprecipitation of either TCR- $\alpha$  mutant with the anti-BiP antibody (represented in Fig. 3, *bottom panel*), most likely due to the fact that a small fraction of the cell lysate (1/100) was used for immunoprecipitation in order to insure that the antibody was saturating.

If retention of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  is a consequence of their stable interaction with BiP, it would require that the majority of all retained molecules be bound to BiP. Densitometric scanning of the 2-D gels (Fig. 3, *top* and *bottom panel*) indicated that the ratio of TCR- $\alpha$  variants and coprecipitated BiP after 16 h of labeling was on the order of 4:1. However, in order to accurately quantitate the fractional labeling of the total protein populations, the relative half-lives of these two proteins must be considered. We have experimentally determined that the half-lives of TCR- $\alpha_t$  in COS cells and TCR- $\alpha_{GPI}$  in COS or CHO cells are  $\sim 14$  and 8 h, respectively, whereas that of BiP is  $>50$  h in both cell lines (data not shown). By taking into account the measured intensities of the labeled proteins corrected for the number of methionines in TCR- $\alpha_t$  or TCR- $\alpha_{GPI}$ , and BiP (four and nine, respectively) (Saito et al., 1984; Becker et al., 1985; Munro and Pelham, 1986), in addition to the relative half-lives of these proteins, the calculated ratio of TCR- $\alpha_t$  or TCR- $\alpha_{GPI}$  to BiP at steady state is  $\sim 1:1$  to  $2:1$ . Thus, ER retention of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  could be accounted for by their stoichiometric interaction with BiP. Throughout this study, labeling periods of 4 h or less were used resulting in a higher ratio of labeled  $\alpha$  chain variant to BiP.

#### *Chelation of Ca<sup>2+</sup> In Vitro Results in Dissociation of TCR- $\alpha$ Variants from BiP*

To examine the nature of the interaction between soluble and lipid-linked forms of TCR- $\alpha$  and BiP, we used various biochemical manipulations in an attempt to disrupt this stable association in whole cell lysates. Cells expressing TCR- $\alpha_t$  were pulse labeled and solubilized in the presence of various reagents, and labeled TCR- $\alpha_t$  was isolated by immunoprecipitation. ATP has been shown to cause the release of BiP from associated proteins such as immunoglobulin heavy chain (Munro and Pelham, 1986), while apyrase, a phosphodiesterase, depletes levels of ATP, and stabilizes BiP binding (Kassenbrock et al., 1988). Since the ER is known to sequester Ca<sup>2+</sup>, we wondered whether Ca<sup>2+</sup> availability could influence the amount of BiP coprecipitated with TCR- $\alpha_t$  or TCR- $\alpha_{GPI}$ , thus, we compared the effect of EGTA which chelates Ca<sup>2+</sup>, to that of ATP and apyrase. (Fig. 4, *top* shows the immunoprecipitation of TCR- $\alpha_t$  from untreated or treated lysates. Fig. 4 *bottom* is a densitometric analysis of the relative ratio of BiP to the truncated  $\alpha$  chain from the corresponding gel lanes, where the ratio in untreated samples is taken to be 100%.)

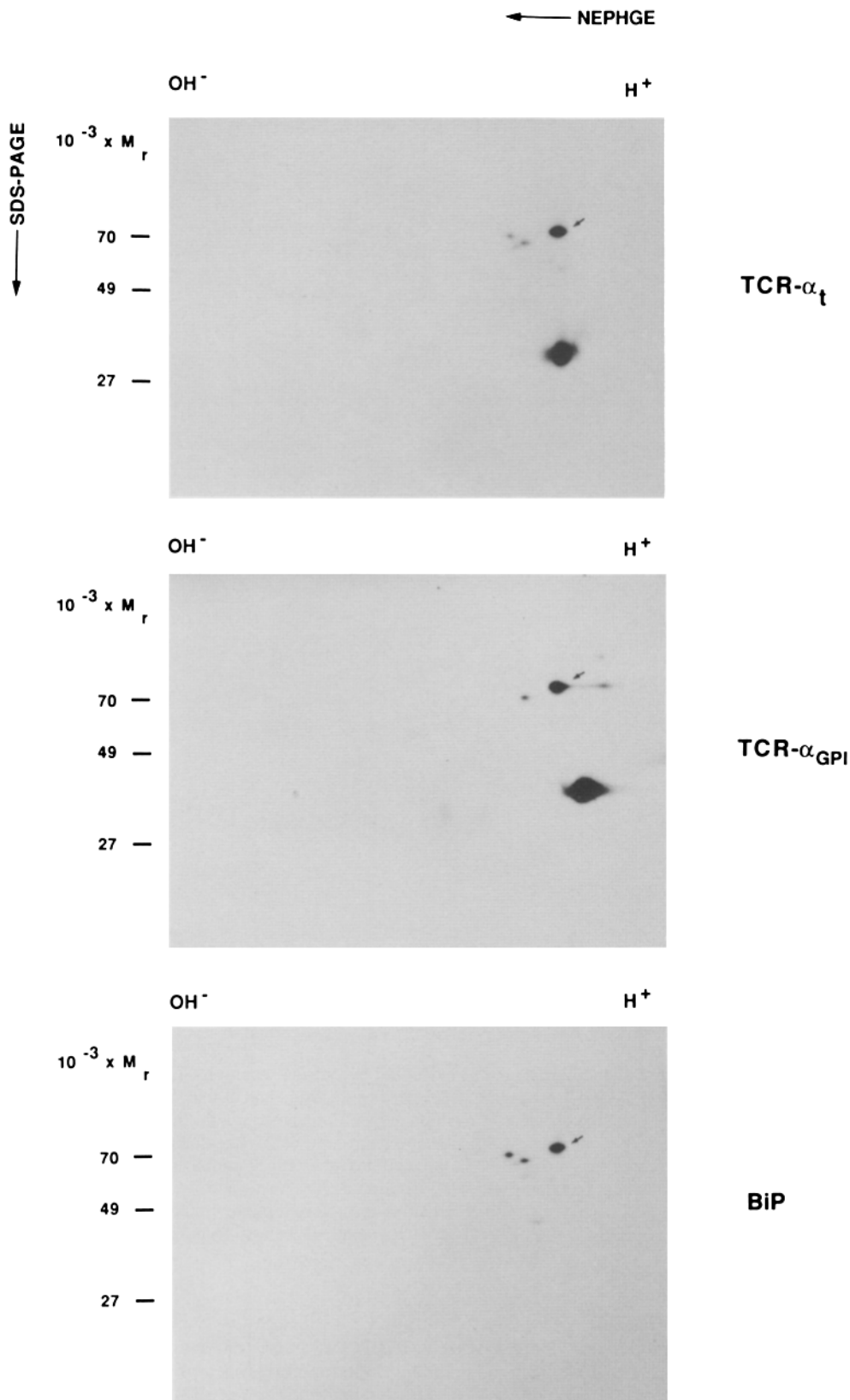


**Figure 2.** Stable retention of TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  in a pre-Golgi compartment as evidenced by endo-H sensitivity. COS cells transiently expressing TCR- $\alpha_t$  (*top panel*) or TCR- $\alpha_{GPI}$  (*bottom panel*) were pulse labeled 36 h after transfection, for 30 min with [ $^{35}$ S]methionine (0.25 mCi/ml) in methionine-free medium at 37°C, and chased in the presence of methionine-containing medium for the times as indicated. Cell pellets were solubilized in 0.5% Triton X-100, and TCR- $\alpha$  variants were isolated from whole cell lysates or culture supernatants by immunoprecipitation with a mAb (A2B4) bound to protein A-agarose. Immunoprecipitates were either untreated (-) or treated (+) with endo H before analysis by SDS-PAGE on 13% acrylamide gels under reducing conditions. The vast majority of both TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  (80%) remain stable intracellularly throughout the chase as an endo H-sensitive form with a minor fraction of TCR- $\alpha_t$  (10%) secreted into the culture medium. Note the coprecipitation of a 78-kD protein with the TCR- $\alpha$  mutants isolated from the cell lysate, though not from the culture supernatants.

Solubilization in the presence of ATP (1 mM) resulted in a 70–80% decrease in the recovery of BiP coprecipitated with TCR- $\alpha_t$  as compared to control, while the presence of apyrase (100 U) during cell lysis stabilized the association of BiP with TCR- $\alpha_t$  (Fig. 4). Interestingly, we have found that the chelation of  $Ca^{2+}$  by EGTA (5 mM) during cell lysis, can result in a 70–80% decrease in the amount of BiP coprecipitated with TCR- $\alpha_t$ . This effect can be blocked by the addition of excess  $Ca^{2+}$  (10 mM) to EGTA-treated lysates (data not shown). Pretreatment of cell lysates with

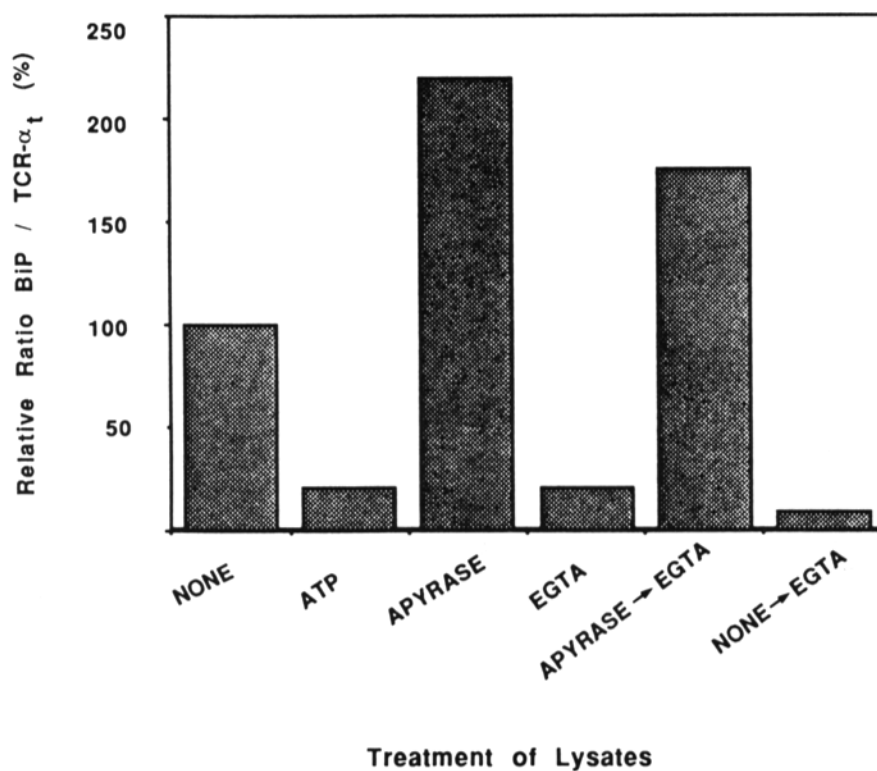
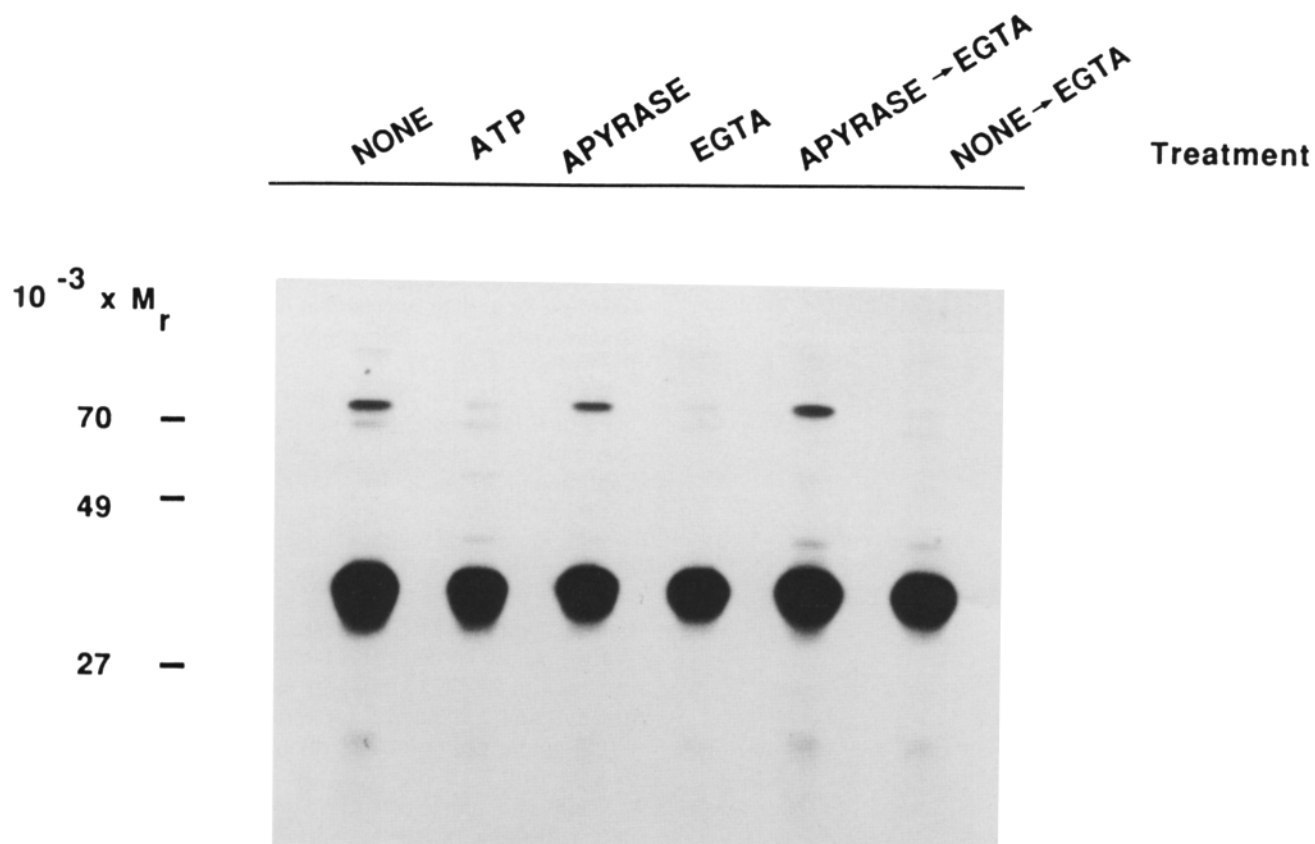
apyrase (5 min at 4°C) blocked the EGTA-induced release of BiP (Fig. 4, *APYRASE* → *EGTA*), suggesting that this effect is dependent upon the availability of ATP. As a positive control, we show that dissociation of BiP can be demonstrated in untreated lysates (incubated for 5 min at 4°C), to which EGTA has been added (Fig. 4, *NONE* → *EGTA*). Furthermore, preincubation of cell lysates with AMP-PNP, a nonhydrolyzable analogue of ATP, abrogates BiP release by EGTA (Fig. 5 A, *AMP-PNP* → *EGTA*).

While the addition of EGTA resulted in the release of BiP

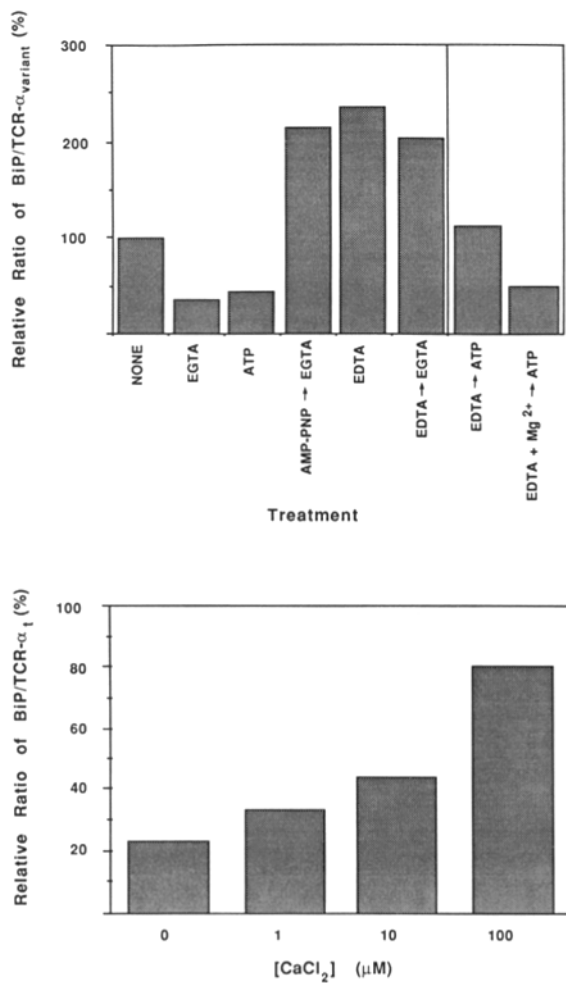


**Figure 3.** Migration of a 78-kD protein coprecipitated with TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  is identical to that of BiP on two-dimensional gel electrophoresis. Transiently transfected COS cells expressing TCR- $\alpha_t$  (*top panel*) or CHO cells expressing TCR- $\alpha_{GPI}$  (*middle panel*), were labeled for 16 h at 37°C with [ $^{35}$ S]methionine (0.5 mCi/ml) in medium that was nine parts methionine-free and one part complete medium containing unlabeled methionine; 10% FBS was included in the labeling medium. Cell pellets were extracted in 0.5% Triton X-100 and TCR- $\alpha_t$  or BiP isolated from whole cell lysates by immunoprecipitation with the mAbs A2B4 and anti-BiP, respectively (*top and bottom panel, respectively*), from COS cells, whereas TCR- $\alpha_{GPI}$  was isolated in the middle panel from CHO cells. Immunoprecipitates were analyzed by NEPHGE using ampholytes (pH, 3–10) in the first dimension, and then analyzed by SDS-PAGE in the second dimension on 13% acrylamide gels under reducing conditions. The arrows indicate BiP in each of the panels. Immunoprecipitation of COS or CHO cell lysates with an irrelevant antibody reveals that the 78-kD spot is specific to the anti-BiP antibody, whereas the minor spots adjacent to BiP are nonspecific. Superimposition of autoradiograms aligning the origins and nonspecific spots show that the BiP coprecipitated with TCR- $\alpha_t$  and TCR- $\alpha_{GPI}$  are identical.

**Figure 4.** Chelation of  $Ca^{2+}$  in whole cell lysates results in the dissociation of BiP from TCR- $\alpha_t$ . Transiently transfected COS cells expressing TCR- $\alpha_t$  were pulse-labeled for 4 h with [ $^{35}$ S]methionine (0.25 mCi/ml) in methionine-free medium at 37°C. Cell pellets were solubilized in freshly prepared lysis buffer (0.5% Triton X-100) without additions, or with addition of either ATP (1 mM, pH 7.6), apyrase (200 U/ml), or EGTA (10 mM, pH 7.6), and incubated for 10 min on ice. Pretreatment of lysates with apyrase for 5 min on ice was fol-



lowed by the addition of EGTA (*APYRASE* → *EGTA*), and incubated for an additional 10 min on ice; untreated lysates were held for 5 min on ice after which EGTA was added and incubated for an additional 10 min as indicated by NONE → EGTA, serving as a positive control. The detergent-insoluble material was pelleted by centrifugation for 15 min at 4°C. TCR- $\alpha_t$  proteins were isolated from whole cell lysates by immunoprecipitation with A2B4 bound to protein A-agarose and analyzed by SDS-PAGE on 13% acrylamide gels.



**Figure 5.** Release of BiP from TCR- $\alpha$  in whole cell lysates is dependent upon the availability of Mg<sup>2+</sup>, hydrolyzable ATP, and Ca<sup>2+</sup> concentrations. COS cells transiently expressing TCR- $\alpha_i$  were pulse labeled with [<sup>35</sup>S]methionine for 4 h at 37°C, after which the cells were harvested. (A) Cells were lysed in the absence (NONE) or presence of EGTA (5 mM) or ATP (1 mM) and incubated for 10 min on ice. Pretreatment of cell lysates with AMP-PNP (1 mM) for 5 min on ice was followed by the addition of 5 mM EGTA (AMP-PNP  $\rightarrow$  EGTA) for an additional 10 min on ice. The requirement for Mg<sup>2+</sup> was examined by lysing the cells in the presence of EDTA (5 mM), incubating the lysates on ice for 5 min, followed by the addition of 5 mM EGTA (EDTA  $\rightarrow$  EGTA) or 1 mM ATP (EDTA  $\rightarrow$  ATP) for an extended 10 min. Finally, cell lysates from CHO cells expressing TCR- $\alpha_{GPI}$  were pretreated in the presence of EDTA plus an excess of Mg<sup>2+</sup> (10 mM MgCl<sub>2</sub>) for 5 min, followed by addition of 1 mM ATP (EDTA + Mg<sup>2+</sup>  $\rightarrow$  ATP). (B) Cells were lysed in the presence of 1 mM ATP and added CaCl<sub>2</sub> at the concentrations indicated (0, 1, 10, 100  $\mu$ M), and incubated for 10 min on ice. The detergent-insoluble material was pelleted by centrifugation for 5 min at 4°C and TCR- $\alpha$  was isolated from cell lysates by immunoprecipitation with A2B4 bound to protein A-agarose and analyzed by SDS-PAGE on 13% acrylamide gels under reducing conditions.

from TCR- $\alpha$ , the addition of EDTA had the opposite effect, resulting in enhanced BiP association. Treatment of lysates with EDTA (5 mM) prevented the dissociation of BiP due to the subsequent addition of EGTA (EDTA  $\rightarrow$  EGTA), or ATP (EDTA  $\rightarrow$  ATP). This suggested that Mg<sup>2+</sup> might be required for the ATP-dependent release of BiP from TCR- $\alpha$ . This was directly tested by first adding EDTA in the presence of excess Mg<sup>2+</sup> (10 mM) before the addition of ATP to the lysates. When this was done, the ATP-induced release of BiP was reconstituted (Fig. 5 A, EDTA + Mg<sup>2+</sup>  $\rightarrow$  ATP). Taken together, these data suggest that the ability of Ca<sup>2+</sup> chelation to dissociate TCR- $\alpha$ -BiP complexes requires both Mg<sup>2+</sup> and the hydrolysis of ATP.

We have ruled out the possibility that EGTA affects the release of BiP by merely mimicking the addition of ATP, for example, by increasing ATP levels in cell lysates due to the inhibition of ATPases. This was examined by measuring the levels of ATP hydrolysis in control and untreated cell lysates by addition of  $\gamma$ -<sup>32</sup>P ATP and adsorption of nucleotide by activated charcoal after various periods of incubation at 4°C. We found that over a 30-min period, ATP hydrolysis was the same in control and EGTA-treated samples (data not shown). We were able to detect an 80% release of BiP in lysates treated with EGTA 15 min after solubilization and immunoprecipitation combined (shorter periods were not practicable), demonstrating that the dissociation effected by EGTA was rapid.

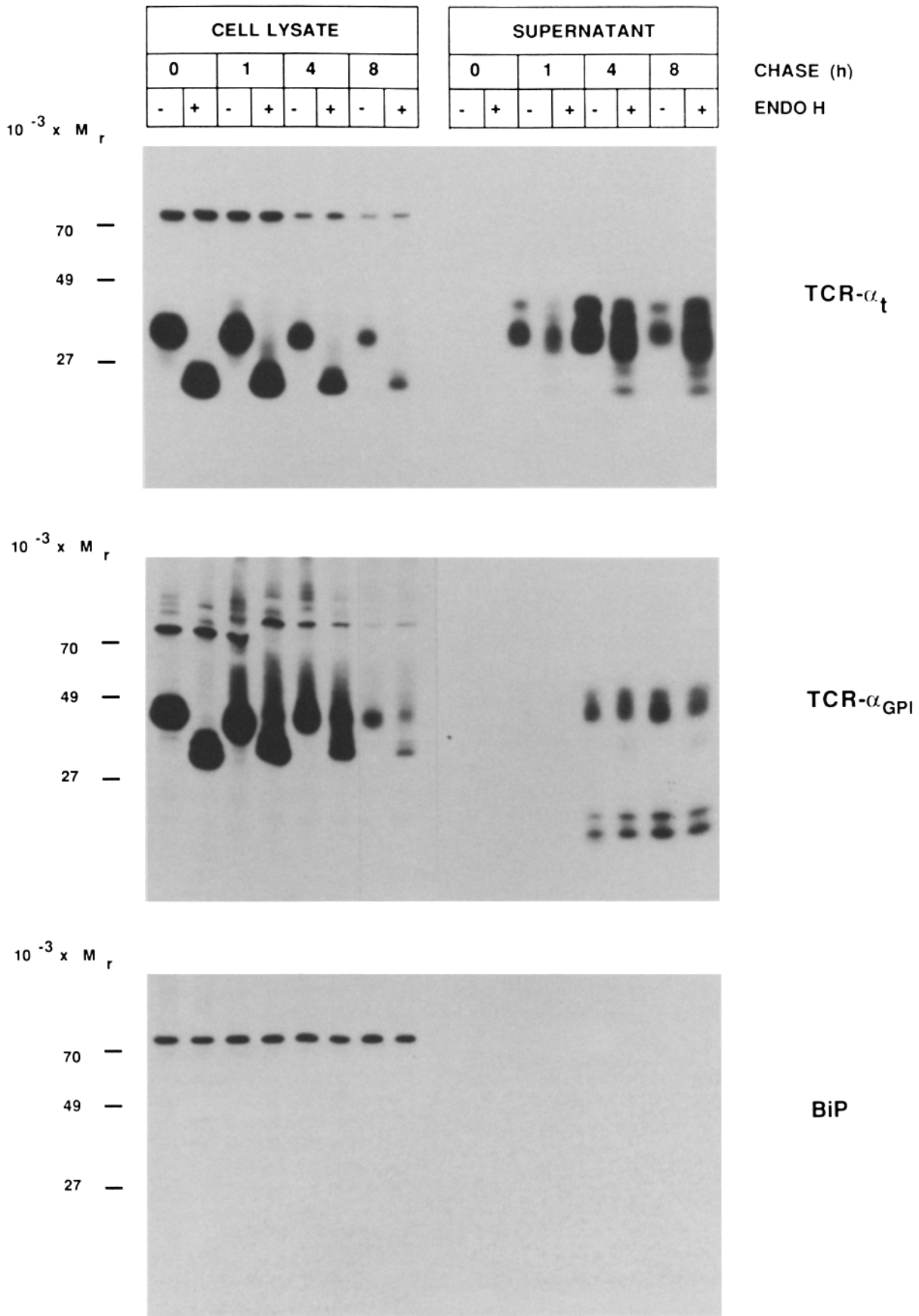
While ATP appeared to be a requirement for the EGTA-induced release of BiP in cell lysates, the reverse was also true in that Ca<sup>2+</sup> acted to antagonize the ability of ATP to dissociate BiP from TCR- $\alpha_i$  (Fig. 5 B). Titration of CaCl<sub>2</sub> in cell lysates containing 1 mM ATP revealed that in the absence of added CaCl<sub>2</sub> in cell lysates, 20% of the BiP coprecipitated with TCR- $\alpha_i$  was recovered relative to the control samples not treated with CaCl<sub>2</sub> or ATP; increasing concentrations of CaCl<sub>2</sub> to 100  $\mu$ M can antagonize the ATP-induced release of BiP, such that 80% of coprecipitated BiP remains associated with TCR- $\alpha_i$ . We have consistently found that the effects of all these in vitro manipulations on the binding of BiP to the membrane-associated TCR- $\alpha_{GPI}$  are indistinguishable from those observed for the truncated form.

### Release from ER Retention in Intact Cells

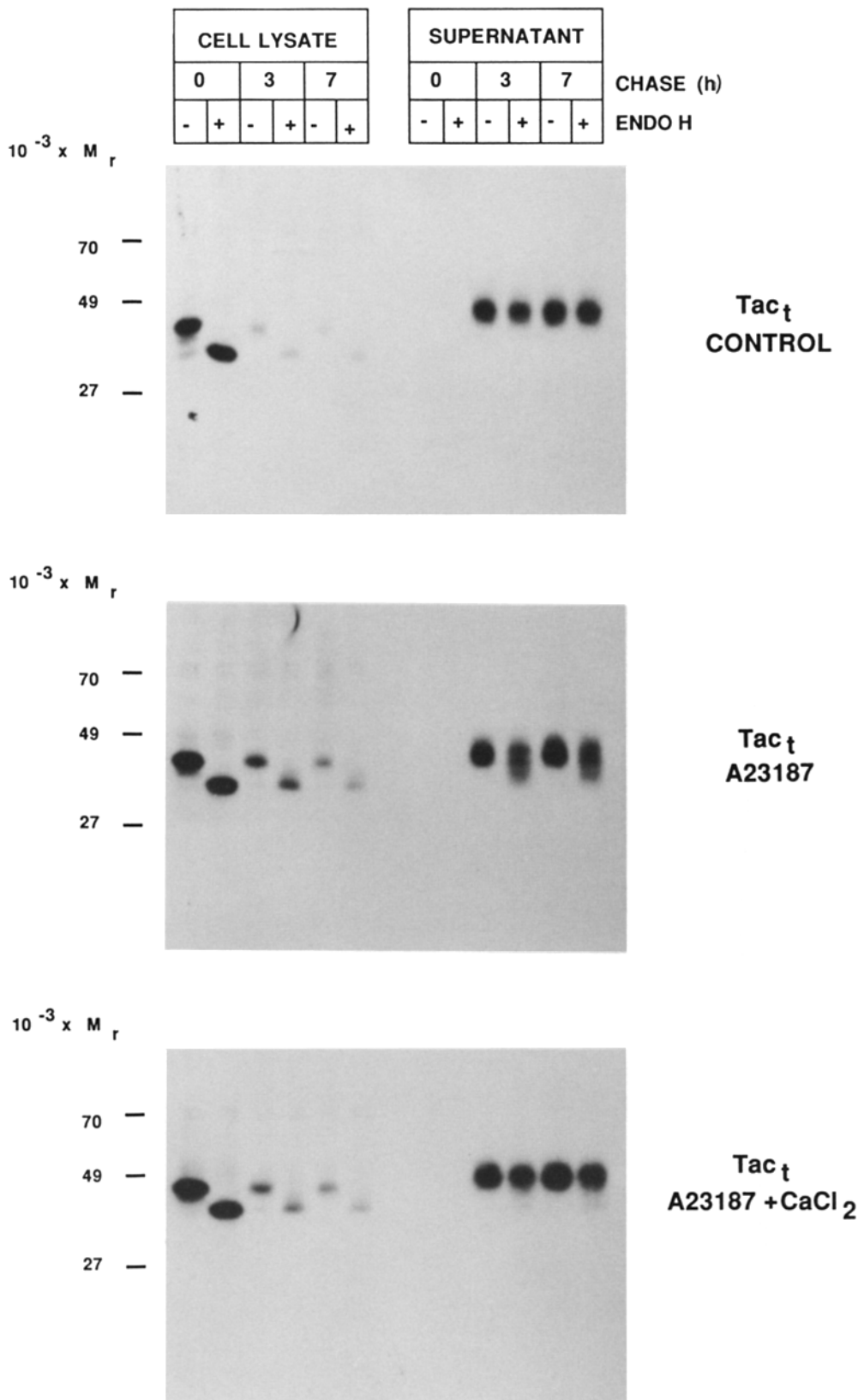
Based on the observation that a reduction of Ca<sup>2+</sup> levels in vitro could result in the release of TCR- $\alpha_i$  from BiP, we examined whether a correlate might be found in vivo. We predicted that if Ca<sup>2+</sup> levels within the ER lumen could be lowered sufficiently, the mutant TCR- $\alpha$  chains would dissociate from BiP and escape from the ER, resulting in the secretion of TCR- $\alpha_i$  and the surface expression of TCR- $\alpha_{GPI}$ . To test this hypothesis, we initially used the Ca<sup>2+</sup> ionophore, A23187, to lower ER Ca<sup>2+</sup> by allowing Ca<sup>2+</sup> (and

**Figure 6.** A23187-induced movement of TCR- $\alpha_i$  and TCR- $\alpha_{GPI}$  out of the ER. COS cells transiently expressing TCR- $\alpha_i$  (top panel) or TCR- $\alpha_{GPI}$  (middle panel) were pulse labeled for 30 min with [<sup>35</sup>S]methionine in methionine-free medium and then incubated with medium containing unlabeled methionine in the absence or presence of A23187 (5  $\mu$ M) at 37°C for the times as indicated. Cell pellets were extracted with 0.5% Triton X-100 and protein immunoprecipitated from whole cells lysates and supernatants with a mAb (A2B4) recognizing the TCR- $\alpha$  variant in the top and middle panels, or an anti-BiP antibody in the lower panel (BiP was isolated from cells expressing TCR- $\alpha_i$ ).





Immunoprecipitates were either untreated (-) or treated (+) with endo H and analyzed by SDS-PAGE on 13% acylamide gels under reducing conditions. Compare the intracellular fate of the TCR- $\alpha$  variants in untreated controls (Fig. 2) with that observed in A23187-treated cells. The fate of BiP in untreated cells is identical to that seen in the presence of A23187.



*Figure 7.* A23187 does not alter the rate of Tac<sub>t</sub> secretion in transiently transfected COS cells. Cells were pulse labeled with [<sup>35</sup>S]methionine for 30 min at 37°C, and then chased in methionine-containing medium in the absence or presence of A23187 (5 mM), or A23187 with added CaCl<sub>2</sub> (20 mM). Tac<sub>t</sub> from cell lysates and supernatants were immunoprecipitated with the mAb 7G7. Immunoprecipitates were either untreated (–) or treated (+) with endo H and analyzed by SDS-PAGE on 13% acrylamide gels under reducing conditions.

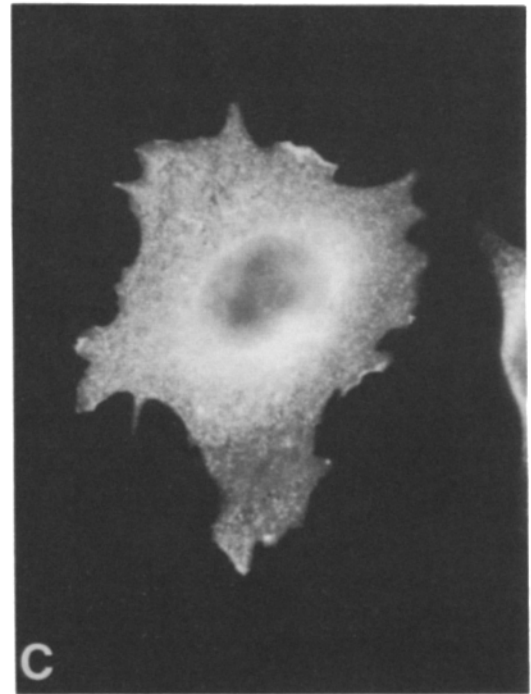
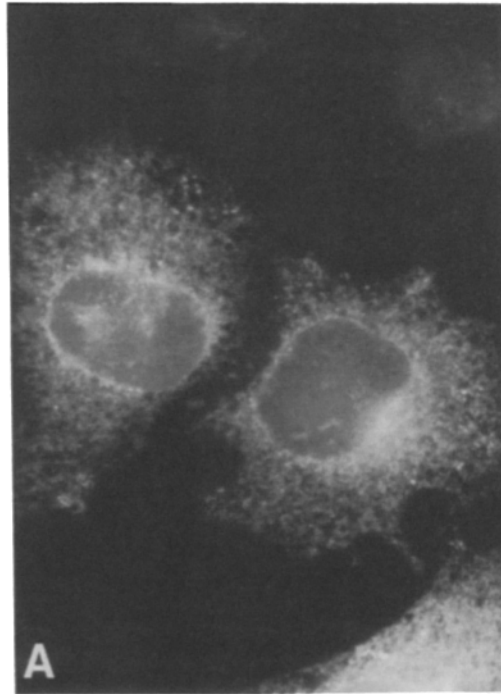
Mg<sup>2+</sup> to a lesser extent) to flow down its concentration gradient into the cytosol (Reed, 1982); it has been inferred that the majority of intracellular Ca<sup>2+</sup> is stored in the ER (Gerok et al., 1990) and that intraluminal Ca<sup>2+</sup> levels are

high with respect to cytosolic levels which range between 0.1 and 0.2 μM (Carafoli, 1987). We found that cells pulse labeled and chased in the presence of A23187 (5 μM) secreted up to six times more TCR-α<sub>i</sub> (Fig. 6, top panel) as com-

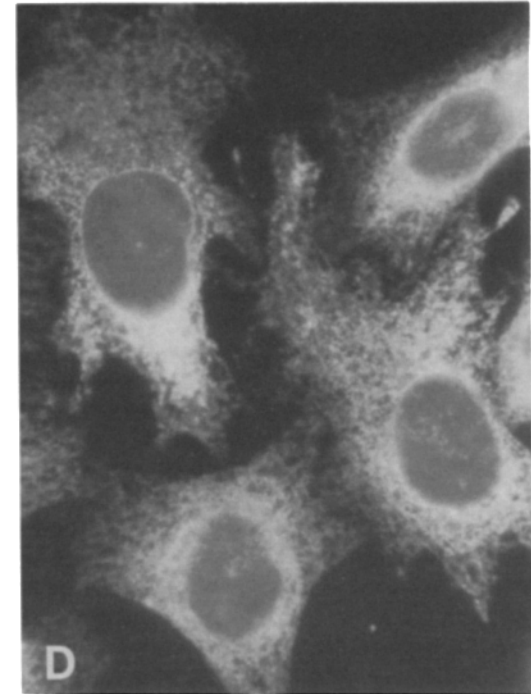
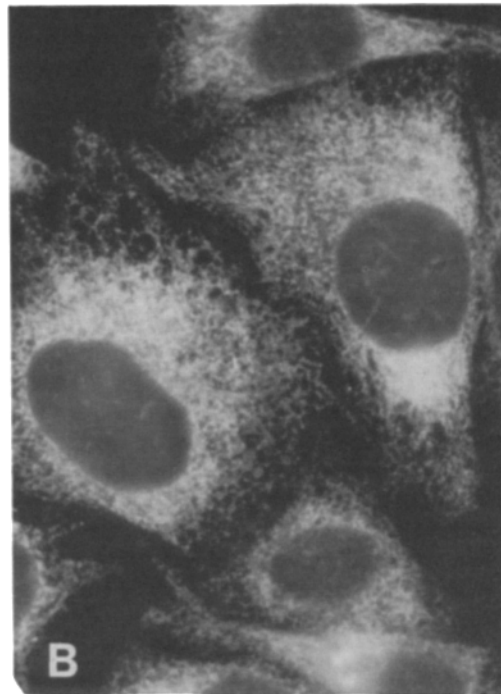
CONTROL

A23187

A2B4



anti-BiP



**Figure 8.** Redistribution of TCR- $\alpha_{GPI}$ , but not BiP, in cells treated with A23187. CHO cells stably expressing TCR- $\alpha_{GPI}$  were incubated with 50 mM  $NH_4Cl$  and 1  $\mu g/ml$  cycloheximide in the absence or presence of A23187 (2.5  $\mu M$ ) for 3 h at 37°C and were prepared for immunofluorescence as previously described (Lippincott-Schwartz et al., 1990). Briefly, cells were fixed in 3% formaldehyde in PBS, and permeabilized in 0.1% saponin. Coverslips were incubated with either an anti-TCR- $\alpha$  mAb A2B4 (A and C), recognizing TCR- $\alpha_{GPI}$ , or, with an anti-BiP mAb (B and D), followed by fluorescently labeled secondary antibodies (rhodamine-conjugated goat antimouse and fluorescein-labeled goat antirat) against the respective species isotypes. In the absence of ionophore, TCR- $\alpha_{GPI}$  and BiP (A and B, respectively) are both distributed in a reticular network throughout the cytoplasm as well as staining of the nuclear envelope characteristic of the ER. After a 3-h treatment with A23187, TCR- $\alpha_{GPI}$  has redistributed to the cell surface (C), whereas BiP (D) appears indistinguishable from controls with no loss in fluorescence intensity.

pared with the untreated controls (Fig. 2, *top panel*) over an 8-h period. After 1 h in A23187, 5% of TCR- $\alpha_i$  expressed transiently in COS cells was present in the culture medium as an endo H-resistant form, whereas in the control no secreted protein was detectable. By 8 h 60% of labeled protein appeared in the culture supernatant in the presence of ionophore, in contrast to only 10% in untreated cells. Exit from the ER appeared to be the rate-limiting step in the movement of this protein throughout the secretory pathway, since all TCR- $\alpha_i$  immunoprecipitated from cell lysates was endo H-sensitive. Some loss of total TCR- $\alpha_i$  was observed in these experiments, most likely due to degradation. Likewise, COS cells expressing the membrane-associated TCR- $\alpha_{GPI}$  were pulse labeled and chased in the presence of A23187 (5  $\mu$ M). We observed the appearance of endo H-resistant forms present intracellularly during the first hour (Fig. 6, *middle panel*), running as a smear between the 70- and 49-kD markers as well as at 43 kD. We believe that the increasing amount of endo H-resistant material in the cell lysates represents protein transported to the cell surface (refer to Figs. 8 and 10). In contrast to untreated controls (Fig. 2, *bottom panel*), ionophore treatment over an 8-h period resulted in a continued loss of endo H-sensitive TCR- $\alpha_{GPI}$  ( $\sim$ 10% remaining), accompanied by a coordinate loss of coprecipitated BiP similar to that observed with the truncated TCR- $\alpha$ . Approximately 20–30% of precipitable TCR- $\alpha_{GPI}$  is recovered from the culture medium; the higher molecular weight form isolated from the culture supernatant may result from the clipping of the GPI anchor. A significant amount of TCR- $\alpha_{GPI}$  is degraded; whether this occurs at the surface or intracellularly has not been established. However, we do know that degradation is not likely to occur within the ER-Golgi system since TCR- $\alpha_{GPI}$  is stable in the presence of  $Ca^{2+}$  perturbation and brefeldin A, which redistributes Golgi membrane and contents to the ER thereby preventing movement of protein out of the ER (Lippincott-Schwartz, 1989).

Notably, there is an absence of coprecipitated BiP with TCR- $\alpha_i$  and TCR- $\alpha_{GPI}$  isolated from the culture medium of A23187-treated cells. We have found that ionophore treatment does not yield secretion of newly synthesized BiP, evidenced by directly immunoprecipitating cell lysates and culture supernatants with the anti-BiP antibody (Fig. 6, *bottom panel*). This is in marked contrast to a previous report by Booth and Koch (1989) which indicated that A23187 induced the secretion of various ER resident proteins including BiP. Furthermore, immunoblotting of cell lysates and supernatants from cells treated with A23187 for 4–8 h revealed that the entire population of BiP remains intracellular in CHO or COS cells expressing the TCR- $\alpha$  variants (data not shown). Thus, A23187 treatment causes intracellular dissociation of TCR- $\alpha$  variants from BiP.

One explanation for the enhanced secretion of TCR- $\alpha_i$  and surface expression of TCR- $\alpha_{GPI}$  might be the accelerated movement of all proteins throughout the secretory pathway in response to  $Ca^{2+}$  ionophore. To address this issue, we have examined the effect of A23187 on a normally secreted protein, utilizing a truncated soluble form of the human IL-2 receptor  $\alpha$  chain (Tac; Bonifacino et al., 1990b) which is efficiently secreted with a half-time of 1 h when expressed transiently in COS cells (Fig. 7). The addition of A23187 did not alter the rate of transport of this protein through the secretory pathway, although the ability to fully process secreted

protein to an endo H-resistant form was somewhat impaired. However, treatment of cells with A23187 in the presence of added  $CaCl_2$  (20 mM) permitted complete processing to occur. These results suggest that the secretion of TCR- $\alpha_i$  is not simply a manifestation of a generalized increase in the rate of protein transport through the secretory pathway.

### ***TCR- $\alpha_{GPI}$ , but Not BiP, Redistributes out of the ER in Response to $Ca^{2+}$ Ionophore***

Although pulse-chase experiments indicated that TCR- $\alpha_i$  and TCR- $\alpha_{GPI}$  were moving through the secretory pathway in response to ionophore while BiP was retained intracellularly, we wondered whether these events occurred in cells where the ER was morphologically and functionally intact. By immunofluorescence microscopy we examined the distribution of BiP in CHO cells expressing TCR- $\alpha_{GPI}$  in the absence and presence of A23187 (5  $\mu$ M, 3 h, 37°C). Use of the lipid-linked TCR- $\alpha$  variant rather than the soluble form for these morphological studies, permitted visualization of “secreted” protein as delivery to the cell surface. Control as well as ionophore-treated cells were incubated with cycloheximide (CHX, 1  $\mu$ g/ml) to inhibit protein synthesis, thereby allowing us to follow the movement of protein over time, in addition to ammonium chloride ( $NH_4Cl$ , 50 mM), which inhibits degradative enzymes within lysosomes potentially allowing us to visualize redistribution of protein to these structures. In untreated control cells, TCR- $\alpha_{GPI}$  and BiP (Fig. 8, *A* and *B*, respectively), are distributed in a reticular and punctate pattern characteristic of the ER, with evident staining of the nuclear envelope. After a 3-h treatment with A23187, the distribution of TCR- $\alpha_{GPI}$  (Fig. 8 *C*) showed a dramatic shift to the plasma membrane. In marked contrast, the distribution of BiP after the same treatment, was indistinguishable from its ER localization in control cells. By this qualitative analysis, no disruption of ER structure was detected. Furthermore, double-labeling experiments with antibodies recognizing TCR- $\alpha$  and a polyclonal antisera directed against ER resident proteins (Louvard et al., 1982), showed that TCR- $\alpha_{GPI}$  traverses the secretory pathway, while ER resident proteins remain localized to the ER, with no apparent breakdown of ER structural integrity in the presence of A23187 for 1, 2, or 4 h (data not shown).

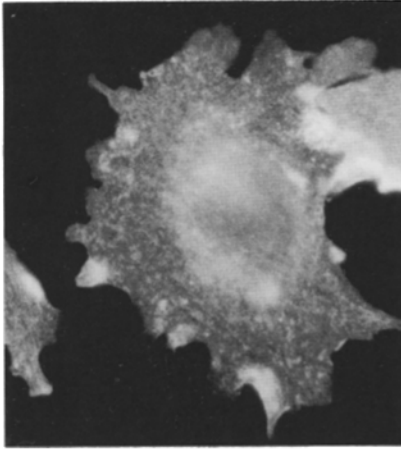
Although we cannot completely assess the degree to which the ER remains functionally intact given A23187 application, we have reason to believe that ionophore treatment does not dramatically disrupt ER function. For example, the secretion rate of a soluble protein such as Tac $_i$  was not altered by A23187 as was shown in Fig. 7. In addition, we have found no change in the rate of ER degradation of the full-length TCR- $\alpha$  chain upon perturbation with ionophore (data not shown).

### ***TCR- $\alpha_{GPI}$ , but Not BiP, Redistributes out of the ER in Response to a Decline in Intraluminal $Ca^{2+}$ Levels***

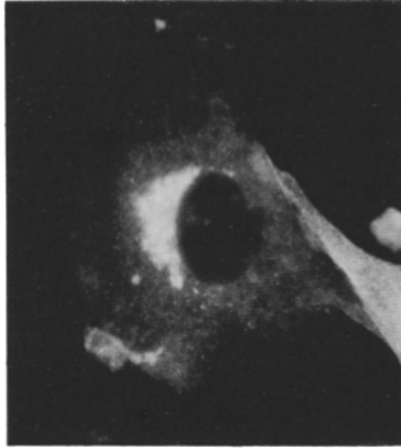
One primary concern in the application of  $Ca^{2+}$  ionophores is its lack of specificity and its undefined effects. To further test that a decline in intraluminal ER  $Ca^{2+}$  levels was responsible for the release of TCR- $\alpha_{GPI}$  from BiP and its exit from the ER, we used the tumor promoter thapsigargin (Tg; Thastrup et al., 1989; Booth and Koch, 1989). Nanomolar concentrations of this drug have been shown to inhibit spe-

Time (h)

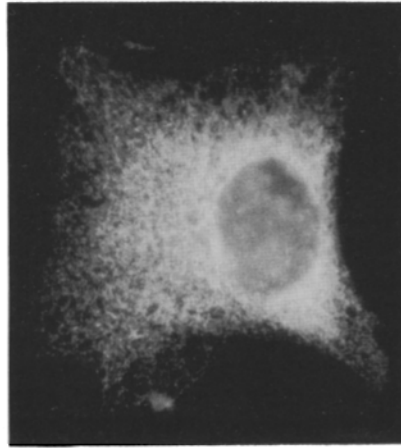
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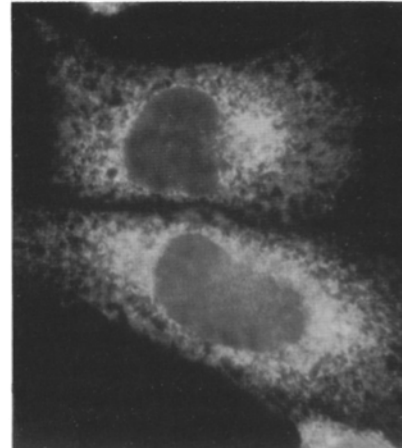
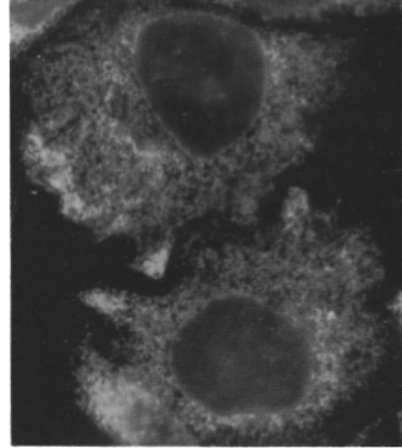
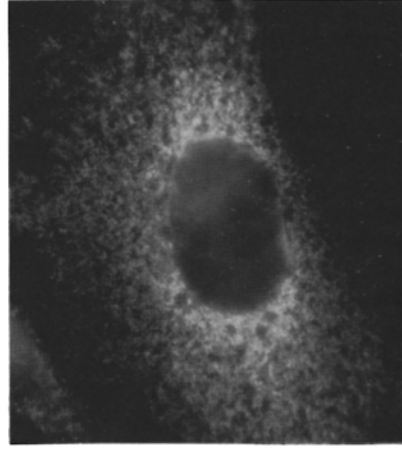
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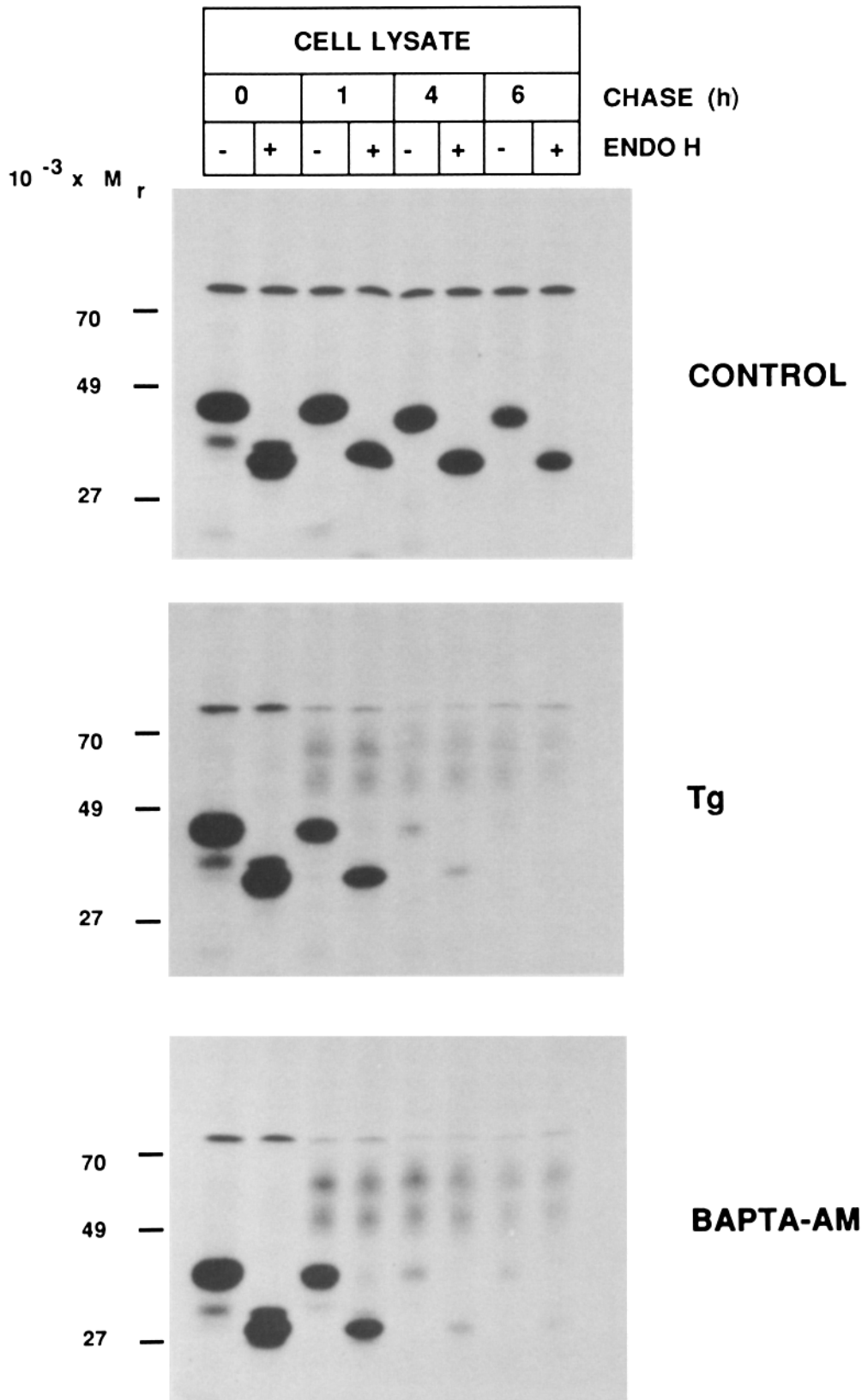


A2B4



anti-BiP

**Figure 9.** Redistribution of TCR- $\alpha_{GPI}$ , but not BiP, out of the ER in cells treated with thapsigargin, an inhibitor of an ER  $Ca^{2+}$ -ATPase. Stably transfected CHO cells expressing TCR- $\alpha_{GPI}$  were incubated with CHX (1  $\mu$ g/ml) in the absence (time = 0 h) or presence of 60 nM thapsigargin (time = 1 or 3 h) at 37°C and prepared for immunofluorescence microscopy as described in Fig. 8. At time = 0, cells labeled with A2B4 show that TCR- $\alpha_{GPI}$  is distributed in a diffuse reticular pattern analogous to that observed when cells are labeled with the anti-BiP antibody. At time = 1 h, TCR- $\alpha_{GPI}$  has redistributed out of the ER into a tight perinuclear structure, by 3 h TCR- $\alpha_{GPI}$  is overwhelmingly on the cell surface, whereas BiP staining in the presence of Tg is unchanged with respect to that seen at time = 0 h.



*Figure 10.* Golgi processing of TCR- $\alpha_{GPI}$  in Tg<sub>1</sub> or BAPTA-AM-treated cells. CHO cells stably expressing TCR- $\alpha_{GPI}$  were pulse labeled for 30 min with [<sup>35</sup>S]methionine in methionine-free medium and then incubated with medium containing unlabeled methionine in the absence (*top panel*) or presence of Tg (100 nM, *middle panel*) or BAPTA-AM (100 nM, *bottom panel*). TCR- $\alpha_{GPI}$  was isolated and analyzed as described in Fig. 6.

cifically an ER Ca<sup>2+</sup> ATPase, resulting in the reduction of ER Ca<sup>2+</sup> levels and a concomitant increase of cytosolic Ca<sup>2+</sup> (Thastrup et al., 1990). CHO cells expressing TCR- $\alpha_{GPI}$ , were either untreated or treated with Tg (50 nM) for 0, 1, or

3 h (Fig. 9) and the distribution of TCR- $\alpha_{GPI}$  and BiP was examined by immunofluorescence microscopy. In untreated controls, TCR- $\alpha_{GPI}$ , like BiP, localizes to the ER, yielding a reticular network throughout the cytoplasm. After 1 h expo-

sure to Tg, the GPI-linked TCR- $\alpha$  has apparently moved into a Golgi-like distribution in at least 90% of the cells with residual reticular staining; by 3 h TCR- $\alpha_{\text{GPI}}$  was overwhelmingly on the cell surface. Double labeling of TCR- $\alpha_{\text{GPI}}$  and the Golgi marker mannosidase II (man II) has shown that TCR- $\alpha_{\text{GPI}}$  colocalizes with man II after 30–60 min in Tg. In marked contrast, BiP remained localized to the ER when cells were incubated with Tg (time = 1 and 3 h) indistinguishable from controls (time = 0 h). Furthermore, we have found that Tg has no effect on the distribution of ER resident proteins recognized by a polyclonal antisera described by Louvard et al. (1982), indicating that structural proteins of the ER inclusive of BiP are not released from this compartment (data not shown).

Consistent with these morphological data we have found that in pulse-chase experiments, movement through the Golgi system can be detected in cells expressing TCR- $\alpha_{\text{GPI}}$  that have been chased for up to 6 h with Tg (50 nM) (Fig. 10). After a 1-h chase period with Tg, two endo H-resistant forms of TCR- $\alpha_{\text{GPI}}$  are observed (Fig. 10, *middle panel*); the highest molecular weight form most likely represents the addition of sialic acid within the trans Golgi (Lippincott-Schwartz et al., 1988), while the lower molecular weight endo H-resistant species represents processing of TCR- $\alpha_{\text{GPI}}$  in the medial Golgi. No endo H resistance of TCR- $\alpha_{\text{GPI}}$  is evident in the untreated controls (Fig. 10, *top panel*).

Finally, one critical possibility that is not addressed by the experiments using A23187 and Tg, is whether the dissociation of soluble and lipid-linked TCR- $\alpha$  variants from BiP and their transport out of the ER is due, not to the decline in luminal ER  $\text{Ca}^{2+}$ , but rather to the increase of cytosolic  $\text{Ca}^{2+}$  induced by both of these reagents. To distinguish between these alternatives, we have used the membrane-permeant  $\text{Ca}^{2+}$  chelator BAPTA-AM. BAPTA is a derivative of EGTA that specifically binds  $\text{Ca}^{2+}$ ; when modified by an acetoxymethyl ester group (AM) it is rendered lipophilic and thus able to cross the plasma membrane as well as intracellular membranes, including that of the ER. Once within the cell, cellular esterases cleave the AM group, whereby the molecule is able to bind  $\text{Ca}^{2+}$  and is no longer membrane permeant (Lew et al., 1982; Grynkiewicz et al., 1985). We have found that TCR- $\alpha_{\text{GPI}}$  in pulse-labeled CHO cells chased in the presence of BAPTA-AM alone (100 nM), is transported through the secretory pathway to the plasma membrane (Fig. 10, *bottom panel*). Within the first hour of exposure to BAPTA-AM, Golgi-processed, endo H-resistant TCR- $\alpha_{\text{GPI}}$  is apparent in contrast to the controls (Fig. 10, *top panel*). The posttranslational modifications of TCR- $\alpha_{\text{GPI}}$  in addition to the kinetics of transport in response to BAPTA treatment, are comparable to that observed with Tg. Common to A23187, Tg, and BAPTA-AM, decreasing amounts of BiP are coprecipitated in accord with decreasing amounts of unprocessed TCR- $\alpha_{\text{GPI}}$ . To confirm that cytosolic-free  $\text{Ca}^{2+}$  levels do not increase in the presence of BAPTA-AM, quantitation of free  $\text{Ca}^{2+}$  levels in control versus BAPTA-AM-treated cells was performed using digital imaging microscopy (Tsien and Harootunian, 1990; Moore et al., 1990). CHO cells expressing TCR- $\alpha_{\text{GPI}}$  were loaded with FURA-2 (see Materials and Methods), in the absence or presence of BAPTA-AM (100 nM) and then treated with Tg (0.2 nM) to induce an increase in cytosolic  $\text{Ca}^{2+}$  levels. Results indicated that when Tg was added to control cells,  $\text{Ca}^{2+}$  levels increased from 170 nM

to millimolar levels within 3 min, whereas in BAPTA-treated cells, free  $\text{Ca}^{2+}$  concentrations did not increase above basal levels. These results support the interpretation that reducing  $\text{Ca}^{2+}$  levels within the ER and not increasing cytosolic  $\text{Ca}^{2+}$ , leads to the dissociation of ER retained TCR- $\alpha$  variants from BiP within this compartment, resulting in their release from ER retention.

## Discussion

Intrinsic to the maturation of newly synthesized proteins destined for secretion or expression on the plasma membrane, are quality control mechanisms by which abnormal and immature proteins are prevented from leaving the ER, either by the retention or degradation of these proteins within this compartment. The finding that two variants of the TCR- $\alpha$  chain (TCR- $\alpha_i$  and TCR- $\alpha_{\text{GPI}}$ ) were retained stably within the ER led us to explore the molecular basis underlying this phenomenon. One possible explanation for the retention of TCR- $\alpha_i$  and TCR- $\alpha_{\text{GPI}}$ , is that these molecules form insoluble aggregates that impede their egress from the ER. However, when we examined this possibility by sucrose gradient analysis, we found that TCR- $\alpha_i$ , as well as TCR- $\alpha_{\text{GPI}}$ , exist as monomers without formation of large aggregated complexes (Suzuki, C., and R. Klausner, unpublished observations). Alternatively, retention might be mediated by a carboxyl-terminal determinant such as that described by Sitia et al. (1990) who have shown that a free cysteine residue within the COOH-terminus of the immunoglobulin  $\mu_c$  chain is responsible for its retention within the ER system. The unpaired cysteine residue involved in the disulfide linkage of the TCR- $\alpha\beta$  heterodimer (Cys<sup>202</sup>), which is maintained in both TCR- $\alpha_i$  and TCR- $\alpha_{\text{GPI}}$ , might function in a similar fashion; however, mutation of Cys<sup>202</sup> to a serine residue in TCR- $\alpha_i$  does not alter its fate as an ER-retained protein (Suzuki, C., and R. Klausner, unpublished observations). Moreover, addition of various carboxyl-terminal sequences (such as the cytoplasmic tail of the IL-2 receptor  $\alpha$  chain) to the same extracellular sequences present in the truncated variant, results in proteins that are nevertheless trapped within the ER (Suzuki, C., and R. Klausner, unpublished observations). These findings suggest that a specific carboxyl-terminal sequence per se, is not responsible for retaining TCR- $\alpha_i$  and TCR- $\alpha_{\text{GPI}}$  in the ER.

Coprecipitation of the abundant ER resident protein, BiP, with both TCR- $\alpha_i$  and TCR- $\alpha_{\text{GPI}}$ , prompted us to question whether ER retention of the TCR- $\alpha$  mutants was mediated by BiP interaction. Previous work by Hendershot et al. (1987) and Pollok et al. (1987) has provided strong evidence that the stable retention of newly synthesized Ig heavy chains within the ER is a consequence of their association with BiP. They have shown that large deletions in either the first constant region domain (CH<sub>1</sub>) or in the variable region (V<sub>H</sub>) of Ig heavy chains result in proteins that do not interact with BiP. The lack of BiP binding is correlated with secretion of CH<sub>1</sub> mutants and the surface expression of V<sub>H</sub> mutants.

One focus of our investigation has been to address whether the association with BiP is responsible for the retention of soluble and membrane-associated proteins, such as TCR- $\alpha_i$  and TCR- $\alpha_{\text{GPI}}$ , respectively. Another aspect has been to examine whether the stable interaction of these ER-retained proteins with BiP can be modulated in vivo, such that release

from ER retention is coincident with release from BiP. The latter issue is of importance not only with respect to understanding ER retention, but also in view of several other proposed functions of BiP, which include the catalysis of protein folding (Pelham, 1989a,b) and the assembly of oligomeric complexes (Pelham, 1989a,b; Rothman, 1989). Identifying the rules by which newly synthesized proteins are not only bound to, but also released from BiP, is critical to examining the role of BiP in normal protein maturation, assembly, and transport within the ER.

Our examination of the in vitro effect of  $\text{Ca}^{2+}$  availability on the association of BiP and the TCR- $\alpha$  variants led us to the finding that chelation of  $\text{Ca}^{2+}$  in whole cell lysates by EGTA results in the release of BiP from TCR- $\alpha_i$  and TCR- $\alpha_{GPI}$ , and that this process is dependent upon the availability of  $\text{Mg}^{2+}$  and hydrolyzable ATP. Conversely, the addition of  $\text{Ca}^{2+}$  can stabilize the association of protein with BiP by antagonizing its ATP-induced release. Kassenbrock and Kelly (1989) have shown that for purified BiP,  $\text{Ca}^{2+}$  can inhibit its ATPase activity such that 0.5 mM  $\text{Ca}^{2+}$  reduces ATP hydrolysis by 70%. Our findings suggest that modulating the ATPase activity of BiP via  $\text{Ca}^{2+}$  levels can serve to regulate the binding to, and release of, proteins associated with BiP. To what extent other proteins retained within the ER exhibit such  $\text{Ca}^{2+}$ -dependent binding to BiP remains to be examined. Preliminary results (Letourneur, F., and R. Klausner, unpublished observations) show that an ER-retained variant of the CD3- $\epsilon$  chain in which the extracellular domain of CD3- $\epsilon$  has been fused to the transmembrane and cytoplasmic domains of the IL-2 receptor  $\alpha$  chain, can coprecipitate BiP in amounts equivalent to those observed for the TCR- $\alpha$  variants. In vitro treatment of lysates revealed that BiP dissociated from this chimeric CD3- $\epsilon$  chain when  $\text{Ca}^{2+}$  is chelated, and that the degree of dissociation is comparable to that induced by ATP. Furthermore,  $\text{Ca}^{2+}$  can antagonize the ability of ATP to release this CD3- $\epsilon$  chimera from BiP as demonstrated for TCR- $\alpha$  variants.

We have found that a correlate to these in vitro observations is that manipulation of  $\text{Ca}^{2+}$  levels via the in vivo application of A23187, can result in the secretion of soluble TCR- $\alpha_i$  and the surface expression of TCR- $\alpha_{GPI}$ , which are otherwise ER retained. In the presence of A23187, BiP is not secreted from COS or CHO cells as determined by pulse-chase experiments and immunoblotting, nor does it appear to redistribute out of the ER by immunofluorescence microscopy, indicating that dissociation from BiP occurs within the ER system. The observation that specific inhibition of an ER  $\text{Ca}^{2+}$ -translocating ATPase by the tumor promoter thapsigargin has the same effect as A23187, supports the idea that the maintenance of elevated luminal  $\text{Ca}^{2+}$  concentrations is necessary for retaining TCR- $\alpha_i$  and TCR- $\alpha_{GPI}$  within the ER. The recent cloning and characterization of the *PMRI* gene in yeast lends further credence to this notion. Rudolph et al. (1989) have shown that the *PMRI* gene product encodes a  $\text{Ca}^{2+}$ -ATPase, and that *pmr1* mutants more efficiently secrete heterologous proteins which are by and large retained in wild-type yeast cells. This defect has been genetically localized to the ER-Golgi transition within the secretory pathway, the site where the release from ER retention would be manifested.

To determine whether the effects seen with A23187 and Tg reflect a decline in luminal ER  $\text{Ca}^{2+}$ , or rather, an increase of cytosolic levels coordinately induced by both of these re-

agents, we utilized a membrane-permeable  $\text{Ca}^{2+}$  chelator BAPTA-AM which is delivered to intracellular compartments as well as to the cytosol. Although we cannot know the extent to which  $\text{Ca}^{2+}$  levels are decreased within the ER, we have found that the presence of 100 nM extracellular BAPTA-AM is sufficient to release the lipid-linked TCR- $\alpha$  variant from the ER and allow for transport through the Golgi as evidenced by the acquisition of carbohydrate modifications associated with the latter.

The molecular details of how  $\text{Ca}^{2+}$  affects the interaction of BiP and target proteins are not known. One formal possibility is that  $\text{Ca}^{2+}$  acts on a target protein bound to BiP by inducing a conformational change that facilitates its release. The alternative explanation, that  $\text{Ca}^{2+}$  acts directly on BiP itself, is supported by previous data indicating that BiP is a  $\text{Ca}^{2+}$ -binding protein (Macer and Koch, 1988) and that  $\text{Ca}^{2+}$  inhibits the ATPase activity of purified BiP (Kassenbrock and Kelly, 1989). Clearly, a variety of stimuli acting through, for example, the  $\text{IP}_3$  receptor (Furuichi et al., 1989; Minery et al., 1989), GTP (Ghosh et al., 1989; Mullaney et al., 1988) or an ER  $\text{Ca}^{2+}$ -ATPase (Damiani et al., 1988; Lytton and MacLennan, 1988), can function to regulate  $\text{Ca}^{2+}$  levels within the ER. In addition, the maintenance of  $\text{Ca}^{2+}$  gradients throughout this complex and dynamic organelle can itself be utilized to regulate the binding and release of proteins from BiP. In so doing, an added level of control can be imposed upon the relationship between the synthesis, maturation, and binding to BiP by proteins at their site of entrance (synthesis) and egress from the ER.

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