

BiP Forms Stable Complexes with Unassembled Subunits of the Acetylcholine Receptor in Transfected COS Cells and in C2 Muscle Cells

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Abstract. We have investigated the role of the immunoglobulin-binding protein (BiP) in the folding and assembly of subunits of the acetylcholine receptor (AChR) in COS cells and in C2 muscle cells. Immunoprecipitation in COS cells showed that α , β , and δ subunits are associated with BiP. In the case of the α subunit, which first folds to acquire toxin-binding activity and is then assembled with the other subunits to form the AChR, BiP was associated only with a form that is unassembled and does not bind α -bungarotoxin. Similar results were found in C2 cells. Although the α and β subunits of the AChR are minor membrane pro-

teins in C2 cells, they were prominent among the proteins immunoprecipitated by antibodies to BiP, suggesting that BiP could play a role in their maturation or folding. In pulse-chase experiments in C2 cells, however, labeled α subunit formed a stable complex with BiP that was first detected after most of the α subunit had acquired toxin-binding activity and whose amount continued to increase for several hours. These kinetics are not compatible with a role for the BiP complex in the folding or assembly pathway of the AChR, and suggest that BiP is associated with a misfolded form of the subunit that is slowly degraded.

THE mechanisms by which oligomeric membrane proteins are assembled in the ER are poorly understood. Resident proteins in the ER have been postulated to facilitate the folding and assembly of newly synthesized proteins; ER proteins may also monitor the folding and assembly pathway, binding to peptides that are misfolded or incorrectly assembled and preventing their exit from the ER (Rose and Doms, 1988; Pelham, 1989; Hurlley and Helenius, 1989). Each of these functions has been ascribed to immunoglobulin-binding protein (BiP)¹ (or glucose-regulated protein 78), a soluble protein resident in the ER that is a member of the hsp 70 family (Haas and Wabl, 1983; Gething et al., 1986; Bole et al., 1986; Pelham, 1986). BiP transiently associates with newly synthesized monomers of immunoglobulin heavy chain and other proteins (Bole et al., 1986; Dorner et al., 1987; Ng et al., 1989), and also binds to mutant proteins that are aggregated or misfolded (Bole et al., 1986; Gething et al., 1986; Dorner et al., 1987; Hendershot et al., 1988).

We have recently used transfected COS cells to investigate the assembly of an oligomeric, ligand-gated ion channel, the nicotinic acetylcholine receptor (AChR) (Gu et al., 1990, 1991b; Yu and Hall, 1991). The AChR, which is composed of five highly homologous subunits, $\alpha_2\beta\gamma\delta$, is assembled in the ER (Smith et al., 1987; Gu et al., 1989a) by a defined

pathway in which the first step is the formation of the heterodimers $\alpha\delta$ and $\alpha\gamma$ (Blount and Merlie, 1989; Blount et al., 1990; Saedi et al., 1991; Gu et al., 1991b). Before heterodimer formation can occur, however, each of the subunits must be correctly folded. This process has been partially characterized for the α subunit which acquires the ability to bind α -bungarotoxin in a posttranslational maturational step that appears to involve disulphide bond formation (Merlie and Lindstrom, 1983; Carlin et al., 1986; Blount and Merlie, 1990). Receptor subunit maturation, which is highly efficient in muscle cells, appears to be much less efficient in transfected COS cells. Thus in muscle cells, approximately one-third of the α subunit acquires toxin-binding activity and is assembled into the AChR (Gu et al., 1989b). In COS cells, in contrast, only a small fraction of the α subunit is converted to the toxin-binding form and undergoes heterodimer formation and assembly (Gu et al., 1991b; Chavez, R. A., and Z. W. Hall, unpublished experiments).

To investigate the possible role of BiP in the folding and assembly of the AChR, we have examined its association with unassembled and assembled subunits of the AChR in both transfected COS cells and in C2 muscle cells. In both cell types we find that BiP is associated with AChR receptor subunits that do not have toxin-binding activity and are not part of a toxin-binding complex. The kinetics of the association of BiP with the newly synthesized α subunit of the AChR in C2 muscle cells suggest that the complex does not play a role in AChR subunit folding or assembly, but that BiP binds misfolded subunits that are retained in a slowly degraded intracellular pool.

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1. *Abbreviations used in this paper:* BiP, immunoglobulin-binding protein; AChR, acetylcholine receptor.

Materials and Methods

Cell Culture

COS cells were grown in DME-H16 (Gibco-BRL, Grand Island, NY) containing 10% FBS (Gu et al., 1990). C2 myoblasts were grown in DME-H16 containing 20% FBS and 0.5% chick embryo extract (Inestrosa et al., 1983). Confluent myoblasts were induced to differentiate by replacement of growth medium with DME-H16 containing 5% horse serum. Under these conditions, multinucleated myotubes represented at least 95% of the cells after 2 d in the differentiation medium.

Transfections

COS cells were transfected as previously described (Gu et al., 1990). Briefly, the day before transfection, cells were plated in 60-mm dishes at a density such that the cells were at ~50% confluence at the time of transfection. The cells were then incubated for 4 h with DME-H16 to which had been added 1% heat-inactivated FBS, 0.1 mM chloroquine, the appropriate plasmid, and 0.4 mg/ml DEAE-dextran in that order with thorough mixing. The transfection mixture was replaced for 2 min with 10% DMSO/90% PBS at room temperature. After incubation of the cells overnight in normal growth medium, the transfected cells were replated in 35-mm dishes and assayed the following day. The optimal amount of plasmid to be transfected was determined empirically. A plasmid containing the cDNA for mouse BiP and SV40 origin of replication was obtained from Dr. M.-J. Gething (University of Texas Southwestern Medical Center) (Kozutsumi et al., 1989). The plasmids used for expression of AChR subunits were those previously described (Gu et al., 1990).

Antibodies

A polyclonal antibody raised in rabbits against mouse BiP (Kozutsumi et al., 1989) was the generous gift of Dr. M.-J. Gething. mAb 61 (anti- α subunit), mAb 124 (anti- β subunit), and mAb 88B (anti- δ subunit) were the kind gifts of Dr. J. Lindstrom (University of Pennsylvania) (61 and 124) and Dr. S. Froehner (University of North Carolina). Secondary antibodies were obtained commercially. Coupling of antibodies to CNBr-activated Sepharose was performed according to the maker's instructions (Pharmacia Fine Chemicals, Piscataway, NJ).

Immunoprecipitation of Labeled COS Cells and C2 Extracts

The protocol for labeling of both cell lines is essentially the same. Dishes of cells were incubated in methionine- and cysteine-free DME-H16 for 30 min at 37°C to deplete the endogenous amino acid pool. They were then incubated for the appropriate amount of time in fresh methionine- and cysteine-free DME-H16 supplemented with 2% dialyzed FBS and ³⁵S-methionine/cysteine (Trans ³⁵S label; ICN) at 100 μ Ci/ml for COS cells and 250 μ Ci/ml for C2 cells. Labeling was terminated by washing the cells three times in ice-cold PBS. In pulse-chase experiments, the cells were then incubated at 37°C in normal medium for the appropriate period and then washed three times with ice-cold PBS. In the case of overnight labeling of C2 myotubes, 10 μ M methionine and 10 μ M cysteine were also added to the medium.

Labeled cells were solubilized in 0.1 ml of a buffer containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM sodium tetrathionate, 1 mM N-ethylmaleimide, 1 mM PMSF, 10 U/ml aprotinin, and 20 μ g/ml leupeptin (extraction buffer). After a 10-min incubation at 4°C, the extract was centrifuged to remove insoluble material and the supernatant solution diluted 10-fold with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, and 0.02% sodium azide ("wash buffer"; Kozutsumi et al., 1989). Antibody (1–2 μ l) was then added and the mixture rotated overnight at 4°C. The antibody-antigen complexes were then precipitated for 2 h at 4°C by the addition of either rabbit anti-rat IgG-Sepharose for mAb 61 and mAb 124, rabbit anti-mouse IgG-Sepharose for mAb 88B, or protein A-Sepharose for anti-BiP antibody (Kozutsumi et al., 1989). The beads were then washed twice with 1 ml of wash buffer at room temperature, boiled in SDS sample buffer, separated on a polyacrylamide gel, and fluorographed by conventional means. In one experiment, a crude membrane fraction was prepared from myotubes suspended in solubilization buffer without Triton X-100 by repeated passage of the cells through a 19-gauge needle. The homogenate was then mixed with KCl to a final concentration of 0.25 M and centrifuged at 100,000 g for 15 min at 4°C. The pellet was then solubilized in Triton X-100 buffer as described above.

Results

AChR Subunits Bind BiP in Transfected COS Cells

We have previously shown that COS cells transfected with cDNAs for the subunits of the AChR assemble the AChR and express it on their surface (Gu et al., 1990, 1991). In the course of these experiments, we observed that when extracts of transfected COS cells that had been labeled with ³⁵S-methionine were immunoprecipitated with antibodies specific for subunits of the AChR, a protein of 80 kD was co-precipitated. The precipitation of this band did not depend on AChR assembly, but could be seen in cells transfected with only a single subunit. Thus cells transfected with α subunit cDNA, labeled with ³⁵S-methionine, and immunoprecipitated with mAb 61, which is specific for the α subunit, showed two prominent bands that were not present in extracts immunoprecipitated with a control mAb. A broad band at 40–43 kD corresponded to the processed and unprocessed forms of the α subunit (Chavez and Hall, 1991); the second prominent band was at 80 kD (Fig. 1). The same 80-kD band was also seen when extracts of cells transfected with cDNAs for β and δ subunits were immunoprecipitated with antibodies to β and δ subunits, respectively (Fig. 1).

Because unassembled subunits appear to be retained in the ER (Smith et al., 1987; Blount et al., 1990), we suspected that the coprecipitated band might correspond to BiP. This possibility was tested in several ways. First, because ATP is known to disrupt the association of BiP with other proteins (Munro and Pelham, 1986; Dorner et al., 1987; Kassenbrock et al., 1988), we carried out an immunoprecipitation in the absence and presence of ATP with extracts of cells transfected with α subunit cDNA (Fig. 2). The presence of ATP caused a slight decrease in the amount of α subunit in the immunoprecipitate, but sharply reduced the amount of 80-kD protein (Fig. 2).

Second, we examined the ability of a polyclonal antibody raised against mouse BiP (Kozutsumi et al., 1989) to immunoprecipitate the α subunit. Because the BiP antibody reacts only weakly with simian BiP (Kozutsumi et al., 1989; also see below) we also transfected COS cells with mouse BiP cDNA. COS cells were transfected either with cDNA for mouse BiP, or with both α subunit and mouse BiP cDNA together, and the cells were metabolically labeled with ³⁵S-methionine. Sham-transfected cells were used as a control. Aliquots of the extracts were then immunoprecipitated either with mAb 61 or with an antibody to mouse BiP. The results of this experiment are shown in Fig. 3. As observed earlier, mAb 61 immunoprecipitated the α subunit (40 and 43 kD) and an 80-kD band from cells transfected with α subunit plus BiP cDNA. Also, as previously observed (Kozutsumi et al., 1989), the mouse BiP in transfected COS cells appeared to migrate slightly faster than the endogenous simian BiP. Neither the α subunit nor the 80-kD band was present in mAb 61 immunoprecipitates of sham-transfected cells or of cells transfected with BiP cDNA alone.

When aliquots of the same extracts were immunoprecipitated with anti-BiP antibody, an 80-kD band was seen in all cases except the sham. The band was strongest in the cells transfected with BiP cDNA or with BiP plus α cDNA. In cells transfected with the α subunit and mouse BiP cDNAs, anti-BiP antibodies immunoprecipitated bands at 40 and 43 kD corresponding to the α subunit. These experiments thus

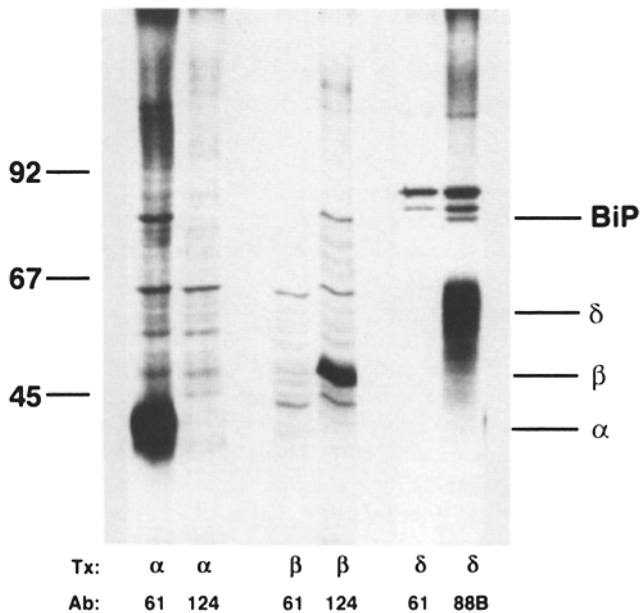


Figure 1. Immunoprecipitation of α , β , or δ subunits expressed in COS cells. COS cells were transfected with cDNA for α , β , or δ subunits, labeled with ^{35}S -methionine, solubilized and immunoprecipitated with the antibodies indicated. mAb 61 is directed against the α subunit, 124 against the β subunit, and 88B against the δ subunit. The immunoprecipitated proteins were separated on a 9% polyacrylamide gel and the gel was exposed to X-ray film.

identify the 80-kD band in the immunoprecipitates from COS cells as BiP, and demonstrate that the α subunit and BiP form a complex.

BiP Is Not Associated with the Mature α Subunit or with AChR Assembly Intermediates

After translation the α subunit undergoes a conformational change that confers toxin-binding activity upon it and allows it to be recognized by a mAb (mAb 35) that does not bind the primary translation product or denatured α subunit (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983; Carlin et al., 1986). The correctly folded α subunit then as-

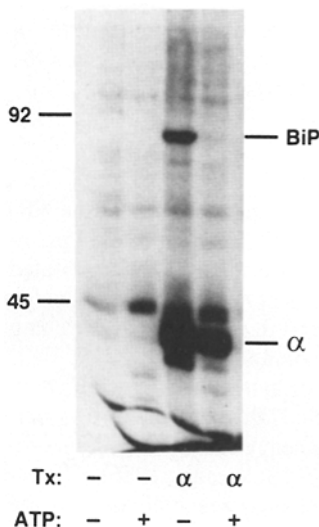


Figure 2. ATP dependence of interaction of α subunit with BiP in COS cells. COS cells were either sham-transfected or transfected with cDNA for the α subunit, labeled with ^{35}S -methionine, solubilized and immunoprecipitated with mAb 61 in the presence and absence of MgATP (10 mM). The immunoprecipitated proteins were then separated on a 7.5% polyacrylamide gel which was then exposed to X-ray film.

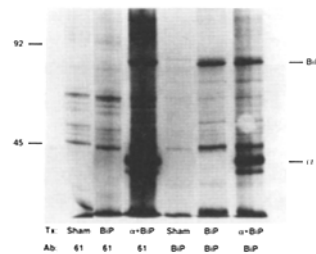


Figure 3. Immunoprecipitation of α subunit in transfected COS cells with anti-BiP antibody. COS cells were either sham-transfected, transfected with α subunit cDNA alone, or transfected with cDNAs for both α subunit and mouse BiP. The transfected cells were labeled with ^{35}S -methionine, solubilized and immunoprecipitated with mAb 61 (anti- α subunit) or with a polyclonal antibody against mouse BiP. The immunoprecipitated proteins were then separated on a 7.5% polyacrylamide gel which was then exposed to X-ray film.

sembles with δ and γ subunits to form heterodimers, and is finally assembled into the intact AChR. To determine at which stages BiP is associated with the α subunit, separate aliquots of extracts of cells transfected with α subunit cDNA were precipitated with mAb 61, which recognizes all forms of α , with mAb 35, with anti-BiP, or with α -bungarotoxin bound to Sepharose. The results (Fig. 4) show, first, that the amount of α subunit that is precipitated by mAb 61 is much larger than that precipitated by mAb 35 or by toxin-Sepharose. This difference is not likely to be due to differences in precipitation in the three cases, as all three are approximately equally efficient in precipitating the intact AChR (results not shown). Thus only a small proportion of the α subunit is correctly folded in transfected COS cells. Second, the results show that BiP is not precipitated by either mAb 35 or by toxin-Sepharose. Thus we conclude that mature, correctly folded α subunit alone does not bind BiP.

We then examined cells that were transfected with α subunit, with α subunit plus δ subunit, or with all four subunits (Fig. 5). The transfected cells were labeled with ^{35}S -methionine, and extracts of them were precipitated with toxin-Sepharose. In no case was BiP seen in the precipitate. Thus, BiP is not associated with α subunit that has toxin-binding activity, either when the subunit is alone or is associated with other subunits.

BiP Is Associated with the α Subunit in C2 Muscle Cells

The α subunit-BiP complex could represent an intermediate in the folding reaction, and thus be a precursor of the mature form of the subunit. Alternatively, BiP could associate with α subunit that is incorrectly folded. Association with BiP in this case would represent an alternative to maturation and as-

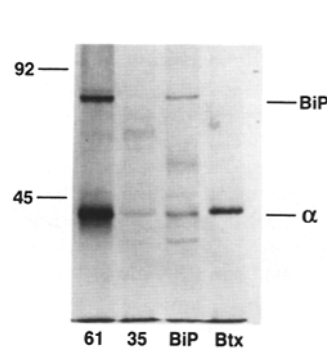


Figure 4. Conformation-dependent interaction of α subunit with BiP. COS cells were transfected with cDNA for the α subunit, labeled with ^{35}S -methionine, solubilized, and immunoprecipitated with mAb 61, mAb 35, anti-BiP antibody or with toxin-Sepharose. The immunoprecipitated proteins were then separated on a 9% polyacrylamide gel which was then exposed to X-ray film.

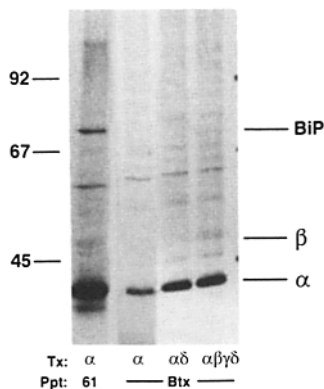


Figure 5. BiP does not bind to toxin binding intermediates or to fully assembled receptor. COS cells were either transfected with α subunit cDNA clone, with cDNAs for both α and δ subunits, or with all four subunits ($\alpha\beta\gamma\delta$). The transfected cells were labeled with ^{35}S -methionine and solubilized in Triton buffer. Labeled cell extracts were precipitated with toxin-Sepharose. An aliquot of extract from cells transfected with α subunit only was immunoprecipitated with mAb 61 (anti- α subunit) as a positive control for the presence of α subunit-BiP complex in the extract. The precipitated proteins were then separated on a 7.5% polyacrylamide gel and the gel exposed to X-ray film.

sociation with other subunits. Because the efficiency of maturation of the α subunit in COS cells is so low, these two alternatives are difficult to distinguish. Therefore, we used the antibody against mouse BiP to investigate the role of BiP in receptor assembly in C2 muscle cells in which maturation is more efficient, and in which the time course of maturation is well-established (Gu et al., 1989a).

In C2 cells subjected to long-term labeling with ^{35}S -methionine, immunoprecipitation with mAb 61, specific for the α subunit, precipitated not only α and β subunits, but also a band with the molecular weight of BiP (Fig. 6, lane 61). A band of the same size was immunoprecipitated with antibodies to BiP (Fig. 6, lane BiP), as were α and β subunits. As with the COS cells, α and β subunits were also precipitated with toxin-Sepharose, but BiP was not. We also found that in C2 cells, the association between the α subunit and BiP is sensitive to ATP (data not shown). These experiments show that in C2 cells, as in COS cells, BiP associates with α subunit that has not acquired toxin-binding activity.

We then carried out a pulse-chase experiment to determine the time course of association of the α subunit with BiP. C2 myotubes were labeled for 10 min, then incubated with unlabeled medium for varying periods of time. Aliquots of the extracts at each time point were precipitated with mAb 61, with anti-BiP and with toxin-Sepharose. The results of this experiment are shown in Fig. 7. In agreement with previous experiments (Gu et al., 1989a), the amount of α subunit immunoprecipitated by mAb 61 decreased rapidly immedi-

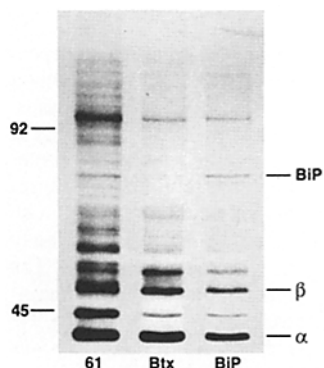


Figure 6. Precipitation of BiP from labeled C2 myotubes. C2 myotubes were labeled for 16 h with ^{35}S -methionine. Triton extracts were incubated with toxin-Sepharose or immunoprecipitated with mAb 61 or anti-BiP antibody. The precipitated proteins were then separated on a 9% polyacrylamide gel which was exposed to X-ray film.

ately after the pulse, then more slowly. At 30 min after the pulse, coprecipitated β subunit first appeared, indicating that assembly of the complete receptor had occurred (Gu et al., 1991b). Because they form more diffuse bands and are more easily degraded, γ and δ subunits cannot be reliably detected by immunoprecipitation after short-term labeling (Gu et al., 1989a). As previously noted (Smith et al., 1987; Gu et al., 1989b), the coincidence in appearance of the β subunit and the decrease in α subunit degradation rate suggests that assembly protects the α subunit from degradation.

When toxin-Sepharose was used as the precipitant, only the α subunit was detected at early times after the pulse (0 and 15 min); at later times the β subunit was also present. After 15 min of chase the amount of α subunit that binds toxin was increased approximately twofold. As β subunit is not present, this increase probably results from the association of the α subunit with γ and δ subunits to form heterodimers (Blount and Merlie, 1989; Gu et al., 1991b).

When association of the subunits with BiP was determined by immunoprecipitation with antibodies to BiP, little or no α or β subunit was immunoprecipitated immediately after the pulse. At 15 min, both were clearly detected. As little association between α and β subunits has occurred at this time, these results suggest that BiP associates independently with each subunit. The amounts of ^{35}S -labeled α and β subunit in the immunoprecipitate continued to increase at an approximately linear rate over the entire 2-h period. In a separate experiment we employed longer chase times to determine the stability of the BiP- α subunit complex. The amount of α subunit immunoprecipitated by BiP antibodies increased over a 6-h period and then began to decrease (Fig. 8). These results show that in C2 myotubes the association between BiP and the α and β subunits is a relatively late event that occurs with a time course that is slow compared to the acquisition of toxin-binding or of AChR assembly. The kinetic relationship between formation of the complex and the acquisition of toxin-binding activity is thus not consistent with a relationship in which the complex is the precursor of the mature subunit. Finally, we examined what proportion of membrane and membrane-associated proteins were BiP associated. Extracts from a crude membrane fraction prepared from myotubes subjected to long-term labeling (Fig. 9) were immunoprecipitated with anti-BiP antibody and compared to the total membrane fraction. Surprisingly few proteins were precipitated by BiP antibody, indicating that BiP is stably associated with a relatively small subset of membrane-associated proteins in muscle cells of which the α and β subunits of the AChR comprise a significant portion.

Discussion

The protein BiP, which is resident in the lumen of the ER, has been previously found in association with several membrane or secreted proteins. It was originally coprecipitated with Ig heavy chains from extracts of myeloma cells in which the Ig light chain is not made and in which the heavy chain accumulates in the ER (Morrison and Scharff, 1975; Haas and Wabl, 1983). In myelomas or hybridomas in which both chains are made and Ig is secreted, BiP was found to associate transiently with monomeric heavy chains or with incompletely assembled IgG molecules (Bole et al., 1986). The association of BiP with heavy chains was increased in the

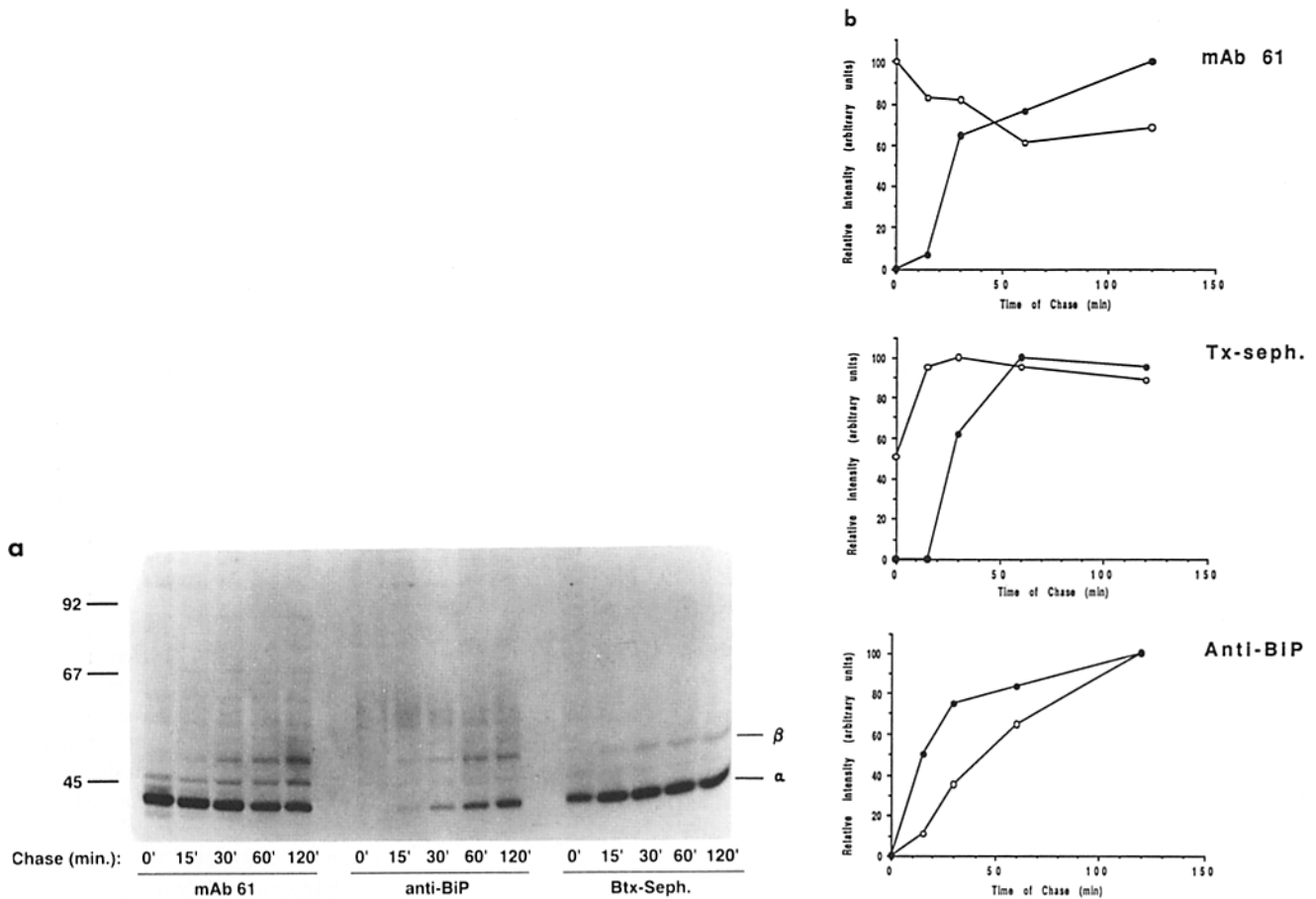


Figure 7. (a) Pulse-chase labeling of C2 myotubes. C2 myotubes were pulse labeled with ^{35}S -methionine for 10 min and then chased in complete medium for the times indicated. Triton extracts of the cells were split into three aliquots and precipitated with toxin-Sepharose or immunoprecipitated with mAb 61 or anti-BiP antibody. The precipitated proteins were then separated on a 9% polyacrylamide gel; the gel was then exposed to X-ray film. The bands corresponding to α and β subunits are marked; the third band, which is nonspecifically precipitated is probably actin. (b) The autoradiogram depicted in a was scanned densitometrically and the data representing the bands of α subunit (open circles) or β subunit (closed circles) plotted as percent of the maximum absorbance observed in each case.

presence of tunicamycin which inhibits glycosylation and renders Ig assembly less efficient. In a second case, wild-type HA proteins in influenza virus-infected cells transiently associate with BiP before their assembly into trimers; misfolded mutant proteins, in contrast, are stably associated with BiP in the ER (Gething et al., 1986). In a third example, the efficiency of secretion of various mutant forms of a tissue plasminogen activator in transfected CHO cells was found to be inversely correlated with their degree of association with

BiP. Also, cotransfection of CHO cells with antisense BiP cDNA and cDNA for poorly secreted tissue plasminogen activator variants increased their rate of secretion (Dorner et al., 1988).

On the basis of these and other experiments, two general

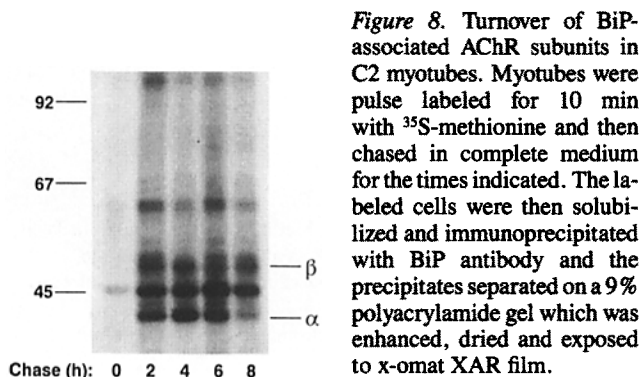


Figure 8. Turnover of BiP-associated AChR subunits in C2 myotubes. Myotubes were pulse labeled for 10 min with ^{35}S -methionine and then chased in complete medium for the times indicated. The labeled cells were then solubilized and immunoprecipitated with BiP antibody and the precipitates separated on a 9% polyacrylamide gel which was enhanced, dried and exposed to x-omat XAR film.

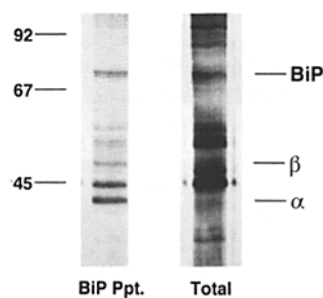


Figure 9. Immunoprecipitation of C2 myotube membrane proteins by BiP antibody. C2 myotubes were metabolically labeled with ^{35}S -methionine for 16 h. The cells were then washed three times with PBS and a crude membrane fraction prepared (see Materials and Methods). The membranes were then solubilized in Triton X-100 (Materials and Methods) and immunoprecipitated with BiP antibody. Approximately 10,000 cpm (0.1% after solubilization) of total solubilized membranes were electrophoresed along with equal amounts of immunoprecipitate. The dried enhanced gel was placed at -70°C next to x-omat XAR film for 4 d.

hypotheses have been proposed for the function of BiP. According to one, binding to BiP represents a transitional stage in the folding or oligomerization of newly synthesized polypeptides; according to the other, BiP binds to polypeptides that are unassembled or incorrectly folded, preventing their exit from the ER and/or facilitating their removal by proteolysis.

The results that we see are most compatible with the second hypothesis. We have found both in transfected COS cells and in C2 muscle cells that BiP is associated with unassembled subunits of the AChR. In the case of the α subunit, only immature forms of the polypeptide are bound to BiP. In a pulse-chase experiment, however, little or no BiP was associated with the α or β subunits at the end of a 10-min pulse, but association steadily increased over a subsequent 6-h time period. As the amount of α subunit with toxin-binding activity was approximately half its final value at the end of the pulse (Fig. 8), the association with BiP that we detect is unlikely to play a role α subunit maturation. The steady increase in α subunit associated with BiP seen in our experiments stands in contrast to the transient association that is seen with endogenous proteins in many other cells.

Stable complexes with BiP such as those seen for the AChR are formed in other systems by proteins that are incorrectly disulfide-bonded. For example, two viral proteins, the influenza hemagglutinin precursor HA0 (Hurtley et al., 1989) and the VSV G protein (Machamer and Rose, 1988; Machamer et al., 1990) are induced to misfold and form incorrect disulphide bonds when tunicamycin blocks glycosylation. These proteins, as well as mutant G proteins with altered glycosylation sites, form aggregates that are stably associated with BiP (Hurtley et al., 1989; Machamer et al., 1990). In the case of HA0, a small amount of the protein made under normal conditions becomes misfolded with abnormal interchain disulfides and remains in the ER where it forms a stable complex with BiP (Hurtley et al., 1989). These observations suggest that the AChR subunits that we find stably associated with BiP in C2 cells have been incorrectly disulphide bonded. The observation by Blount and Merlie (1991) that the α subunit associated with BiP in transfected cells has a slightly different mobility in non-reduced SDS gels than does correctly folded α subunit is consistent with this hypothesis. The very slow formation of the BiP complexes after a pulse chase suggests that some factor other than misfolding may be necessary for complex formation. One possibility is that BiP binds tightly to slowly-forming aggregates of misfolded proteins (Munro and Pelham, 1986; Pelham, 1986).

It should be noted that the immunoprecipitations in all of our experiments detect only high-affinity interactions with BiP. BiP could thus play a role in the folding of the AChR subunits through low-affinity, easily reversible interactions that we do not detect. In a recent paper, Blount and Merlie (1991) examined the association of BiP with AChR subunits in permanently transfected fibroblast lines and in cultures of BC3H-1 cells, a nonfusing muscle cell line. The results that they obtained are similar to those observed here, except that they did not investigate the kinetics of the association between BiP and the α subunit. In contrast to the interpretation that we make, they concluded that BiP was likely to play a role in α subunit maturation. Paulson et al. (1991) have also

reported that α subunit expressed in transfected cells is associated with BiP.

A surprising finding in our experiments was that in C2 cells, the α and β subunits of the acetylcholine receptor were among the most prominent bands immunoprecipitated by an antibody to mouse BiP, even though they constitute only a fraction of a percent of the labeled membrane proteins made by C2 cells (Fig. 9). Previous investigations of BiP have been carried out on myeloma cells or on virally infected or transfected cells, in which the protein associated with BiP is a major protein product of the cell. In these cases, the prominent association of the viral or secreted protein with BiP is compatible with a general role for BiP in binding to polypeptides in the ER. Our results, in contrast, suggest that BiP may play a more specialized role, binding only to particular polypeptides in the cell. The prominence of the α and β subunits in the immunoprecipitates could arise because these proteins are particularly susceptible to misfolding; alternatively, they may be more slowly degraded than other proteins that are bound to BiP.

The significance of association with BiP remains unclear. Much of the newly synthesized α subunit is not assembled into the AChR in C2 cells (Gu et al., 1989a), but is degraded. The rapid degradation of α subunit is essentially complete by 50 min, however (Fig. 8 and Gu et al., 1989a), at a time when association of α subunit with BiP continues. The slow time course of association and the stability of the subunits associated with BiP complex suggest that BiP does not play a role in facilitating the degradation of misfolded subunits. The fate of the α subunit associated with BiP is unknown; presumably it is slowly degraded along with other ER proteins (Lippincott-Schwartz et al., 1988). The half-time of the α subunit-BiP complex (~ 8 h) is comparable to the metabolic half-life of the AChR itself. We have previously observed in pulse-chase experiments a small discrepancy between the amount of α subunit in the assembled AChR and the total amount of α subunit remaining after a long chase period (Gu et al., 1989a); the difference between the two could represent a relatively stable or slowly degraded internal pool of α subunit that is associated with BiP.

In summary, the principal findings of our experiments are: (a) that BiP binds to subunits of the AChR in COS and in C2 cells; (b) that BiP does not bind to the mature (toxin-binding form) of the α subunit; (c) that the kinetics of binding of BiP to the α and β subunits in C2 cells is inconsistent with a role for BiP in maturation of the AChR; and (d) that the α and β subunits of the AChR are prominent components of a small subset of cellular membrane proteins that bind to BiP. On the basis of these results, we suggest that BiP plays a role in neither maturation nor assembly of the AChR subunits. We suggest that association with BiP may represent a pathway for AChR subunit polypeptides that have misfolded, perhaps preventing them thereby from interfering with steps in the assembly pathway.

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References

- Beckman, R. P., L. A. Missen, and W. J. Welch. 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science (Wash. DC)*. 248:850-853.
- Blount, P., and J. P. Merlie. 1989. Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. *Neuron*. 3:349-357.
- Blount, P., and J. P. Merlie. 1990. Mutational analysis of muscle nicotinic acetylcholine receptor subunit assembly. *J. Cell. Biol.* 111:2613-2621.
- Blount, P., and J. P. Merlie. 1991. BiP associates with newly synthesized subunits of the mouse muscle nicotinic receptor. *J. Cell Biol.* 113:1125-1132.
- Blount, P., M. S. McHardy, and J. P. Merlie. 1990. Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell Biol.* 111:2601-2611.
- Bole, D. G., L. M. Hendershot, and J. F. Kearny. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558-1566.
- Carlin, B. E., J. C. Lawrence Jr., J. M. Lindstrom, and J. P. Marlie. 1986. An acetylcholine receptor precursor α subunit that binds α -bungarotoxin but not d-tubocurarine. *Proc. Natl. Acad. Sci. USA*. 83:498-502.
- Chavez, R. A., and Z. W. Hall. 1991. The transmembrane topology of the amino terminus of the α subunit of the nicotinic acetylcholine receptor. *J. Biol. Chem.* 266:15532-15538.
- Dorner, A. J., D. G. Bole, and R. J. Kaufman. 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. *J. Cell Biol.* 105:2665-2674.
- Dorner, A. J., M. G. Kvane, and R. J. Kaufman. 1988. Reduction of endogenous GRP 78 level improves secretion of a heterologous protein in CHO cells. *Mol. Cell. Biol.* 8:4063-4070.
- Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell*. 46:939-950.
- Gu, Y., R. A. Black, G. Ring, and Z. W. Hall. 1989a. A C2 muscle cell variant defective in transport of the acetylcholine receptor to the cell surface. *J. Biol. Chem.* 264:11952-11957.
- Gu, Y., E. Ralston, R. A. Black, C. Murphy-Erdosh, and Z. W. Hall. 1989b. Acetylcholine receptor in a C2 muscle cell variant is retained in the endoplasmic reticulum. *J. Cell Biol.* 109:729-738.
- Gu, Y., A. Franco, Jr., P. D. Gardner, J. B. Lansman, J. R. Forsayeth, and Z. W. Hall. 1990. Properties of embryonic and adult muscle acetylcholine receptors transiently expressed in COS cells. *Neuron*. 5:147-157.
- Gu, Y., P. Camacho, P. Gardner, and Z. W. Hall. 1991a. Identification of two amino acid residues in the epsilon subunit that promote mammalian muscle acetylcholine receptor assembly in COS cells. *Neuron*. 6:879-887.
- Gu, Y., J. R. Forsayeth, S. Verrall, X.-M. Yu, and Z. W. Hall. 1991b. Assembly of the mammalian muscle acetylcholine receptor in transfected COS cells. *J. Cell Biol.* 114:799-807.
- Haas, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature (Lond.)*. 306:387-389.
- Hendershot, L. M., J. Ting, and A. S. Lee. 1988. Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose regulated protein and the role of posttranslational modifications in its binding function. *Mol. Cell Biol.* 8:4250-4256.
- Hurtley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277-307.
- Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland. 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). *J. Cell Biol.* 108:2117-2126.
- Inestrosa, N. C., J. B. Miller, L. Silberstein, L. Ziskind-Conhaim, and Z. W. Hall. 1983. Developmental regulation of 16 S acetylcholinesterase and acetylcholine receptors in a mouse muscle cell line. *Exp. Cell Res.* 147:393-405.
- Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly. 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. *Nature (Lond.)*. 333:90-93.
- Kozutsumi, Y., K. Normington, E. Press, C. Slaughter, J. Sambrook, and M. J. Gething. 1989. Identification of immunoglobulin heavy chain binding protein as glucose-regulated protein 78 on the basis of amino acid sequence, immunological cross-reactivity, and functional activity. *J. Cell Sci. Suppl.* 11:115-137.
- Lippincott-Schwartz, J., L. Yuan, J. S. Bonifacino, and R. D. Klausner. 1988. Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. *Cell*. 54:209-229.
- Machamer, C. E., and J. K. Rose. 1988. Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. *J. Biol. Chem.* 263:5955-5960.
- Machamer, C. E., R. W. Doms, D. G. Bole, A. H. Helenius, and J. K. Rose. 1990. Heavy chain binding protein recognizes incompletely disulfide-bonded forms of vesicular stomatitis virus G protein. *J. Biol. Chem.* 265:6879-6883.
- Merlie, J. P., and J. Lindstrom. 1983. Assembly *in vivo* of mouse muscle acetylcholine receptor: identification of an α subunit species that may be an assembly intermediate. *Cell*. 34:747-757.
- Merlie, J. P., and R. Sebbane. 1981. Acetylcholine receptor subunits transit a precursor pool before acquiring α -bungarotoxin binding activity. *J. Biol. Chem.* 256:3605-3608.
- Morrison, S. L., and M. D. Scharff. 1975. Heavy chain-producing variants of a mouse myeloma cell line. *J. Immunol.* 114:655-659.
- Munro, S., and H. R. B. Pelham. 1986. An hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*. 46:291-300.
- Ng, D. T. W., R. E. Randall, and R. A. Lamb. 1989. Intracellular maturation and transport of the SV5 type II glycoprotein hemagglutinin-neuraminidase: specific and transient association with GRP78-BiP in the endoplasmic reticulum and extensive internalization from the cell surface. *J. Cell Biol.* 109:3273-3289.
- Normington, K., K. Kohno, Y. Kozutsumi, M. J. Gething, and J. Sambrook. 1989. S. cerevisiae encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell*. 57:1223-1236.
- Paulson, H. L., A. F. Ross, W. N. Green, and T. Claudio. 1991. Analysis of early events in acetylcholine receptor assembly. *J. Cell Biol.* 113:1371-1384.
- Pelham, H. R. B. 1986. Speculations on the functions of the major heat shock glucose-regulated proteins. *Cell*. 46:959-961.
- Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257-288.
- Saedi, M. S., W. G. Conroy, and J. Lindstrom. Assembly of Torpedo acetylcholine receptors in *Xenopus* oocytes. *J. Cell Biol.* 112:1007-1015.
- Smith, M. M., J. Lindstrom, and J. P. Merlie. 1987. Formation of the α -bungarotoxin binding site and assembly of the nicotinic acetylcholine receptor subunits occur in the endoplasmic reticulum. *J. Biol. Chem.* 262:4367-4376.
- Yu, X.-M., and Z. W. Hall. 1991. Extracellular domains mediate subunit interactions of the muscle acetylcholine receptor. *Nature (Lond.)*. 352:64-67.