BIP Associates with Newly Synthesized Subunits of the Mouse Muscle Nicotinic Receptor

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Abstract. A slow conformational change in newly synthesized acetylcholine receptor subunits is thought to be a requisite step in the biogenesis of this multisubunit transmembrane glycoprotein. Previously, we demonstrated that this early conformational change within the α-subunit was inefficient and dependent upon disulfide bond formation (Blount, P. and J. P. Merlie. 1990. *J. Cell Biol.* 111:2613–2622). Here we show that newly synthesized acetylcholine receptor subunits and subunit complexes in the muscle-like cell line, BC3H-1, are associated with Bip, a ubiquitous binding protein of the endoplasmic reticulum. Characterization of the Bip/α-subunit complex in stably transfected fibroblasts revealed that Bip associates with newly synthesized unassembled α-subunit and some $\alpha\gamma$ and $\alpha\delta$ subunit complexes. Significantly, Bip does not associate well with the more mature form of the α-subunit containing an intramolecular disulfide bridge. Hence, Bip may play an important role in the conformational maturation and/or editing of unassembled AChR subunits and subunit complexes in vivo.

THE muscle-type nicotinic acetylcholine receptor (AChR),[1] originally isolated from *Torpedo* electric organ, is the best characterized ligand-gated channel and remains the prototype for this family of molecules. This receptor is composed of four different but homologous subunits (Noda et al., 1983) with multiple transmembrane domains assembled around a central channel in a stoichiometry of $\alpha_2\beta\gamma\delta$ (for reviews see Karlin, 1980; Changeux et al., 1984; Claudio, 1989). Several studies have indicated that the two α subunits are not juxtaposed (Wise et al., 1981; Kistler et al., 1982; Zingsheim et al., 1982; Bon et al., 1984) and contribute domains for the two nonequivalent binding sites for competitive antagonists and agonists (Neubig and Cohen, 1979; Haggerty and Froehner, 1981; Kao et al., 1984; Dennis et al., 1986; Sine and Taylor, 1980, 1981; Blount and Merlie, 1989). Studies of the biogenesis of AChR in a muscle-like cell line, BC3H-1, suggested that the α-subunit acquires the ability to bind a snake venom toxin, α-bungarotoxin (BTX), in a time-dependent manner before assembly with other subunits (Merlie and Lindstrom, 1983). We have referred to this conformational change as subunit maturation. Recent studies in which mutant and wild-type subunits were transfected into and expressed in fibroblasts suggested that the disulfide bridging of two cysteines within the α-subunit is required for subunit maturation (Blount and Merlie, 1990). Coexpression of mutant or wild-type α with the δ-subunit in stably transfected fibroblasts demonstrated that unassembled subunits and $\alpha\delta$ complexes containing conformationally immature α-subunit were degraded more rapidly than $\alpha\delta$ complexes containing the conformationally mature α-subunit that binds BTX with high affinity (Blount and Merlie, 1990). Finally, studies on the glycosylation of unassembled and partially assembled AChR subunits in transfected fibroblasts (Blount et al., 1990), and subcellular fractionation of BC3H-1 cells (Smith et al., 1987) suggested that subunit maturation and pentamer assembly occurred in the ER. In sum, these data suggest that the cellular machinery that assists and edits subunit maturation and subunit assembly exists within the ER.

One candidate for assisting (or editing) AChR subunit maturation and assembly is immunoglobulin heavy chain binding protein, Bip (Haas and Wabl, 1983), a resident protein of the ER (Pelham, 1986; Munro and Pelham, 1987). Bip has been found in all cell types examined, and its production is stimulated by several perturbations including inhibition of glycosylation by tunicamycin, calcium ionophores, glucose starvation, and the production of misfolded or mutated proteins (Lee, 1987). Even in unstimulated cells, Bip is expressed at high levels and has been demonstrated to associate transiently with unassembled subunits of some multimeric proteins (Haas and Wabl, 1983; Bole et al., 1986; Hurtley et al., 1989; Ng et al., 1989; Machamer et al., 1990; Hendershot, 1990). Functions proposed for Bip include: participating in the fold-

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; BTX, bungarotoxin; Bip, binding protein.

ing or refolding of misfolded proteins, assisting in the assembly of multimeric proteins, and preventing stress induced aggregation of ER proteins.

Here we demonstrate that Bip binds newly synthesized AChR α and β subunits and subunit complexes in the muscle-like cell line, BC3H-1. Using stably transfected fibroblast cell lines, we provide evidence that Bip associates with the newly synthesized unassembled α, and $\alpha\gamma$ and $\alpha\delta$ assembly intermediates, but does not associate efficiently with the more mature form of the α-subunit that has acquired an intramolecular disulfide bridge. Hence, Bip may be one of the ER proteins that assist in processing or editing of unassembled and partially assembled subunits and subunit complexes.

Materials and Methods

Materials

The protease inhibitor PMSF and *Staphylococcus aureus* cell walls were purchased from Bethesda Research Laboratories (Gaithersburg, MD); tunicamycin and α_2-macroglobulin were purchased from Boehringer Mannheim (Indianapolis, IN); leupeptin, ATP, and β,γ-methyleneadenosine 5′ triphosphate were purchased from Sigma Chemical Co. (St. Louis, MO); β-mercaptoethanol was purchased from Fisher Scientific (Pittsburgh, PA); [^{35}S]methionine (>800 Ci/mmol) was purchased from Amersham Chemical Co. (Arlington Heights, IL). The mAbs mAb61 (Tzartos et al., 1981), and mAb88B (Froehner et al., 1983) were gifts from Drs. Jon Lindstrom (the University of Pennsylvania) and Stanley Froehner (Dartmouth College) respectively. The anti-Bip antibody was a generous gift from Dr. Linda Hendershot (St. Jude Children's Research Hospital) (Bole et al., 1986). Isolation of α-BTX and antibodies to BTX have been previously described (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983).

Cell Growth and Labeling

Growth conditions for BC3H-1 cells (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983), QT-6 cells (Moscovicci et al., 1977; Blount and Merlie, 1989), and the transfection, selection, and maintenance of QT-6 clones expressing the α (Blount and Merlie, 1988), α and γ, and α and δ subunits (Blount and Merlie, 1989) of the mouse AChR have been described. Pulse labeling with [^{35}S]methionine was performed at a specific activity of ~800 Ci/mmol for 5-min pulses, and 10 Ci/mmol for longer pulses.

Immunoprecipitation and Related Methods

Labeled cells were washed twice with PBS 300 μM PMSF at 4°C, scraped from the plates, and pelleted by centrifugation for 10 s in a microfuge. The cell pellet from a single 10-cm dish was extracted with 1 ml PBS, 1-2% Triton X-100, 200 μM leupeptin, and 0.2 U/ml of α_2 macroglobulin for 3–5 min. The cell extracts were centrifuged for 5 min in a microfuge and the supernatant collected. Immunoprecipitations were performed as previously described (Merlie and Sebbane, 1981; Merlie and Lindstrom, 1983) with modifications (Blount and Merlie, 1988). Nonspecific precipitation was assessed by immunoprecipitation with only the second antibody used for mAb61 and anti-Bip, anti-rat IgG. Fig. 3 shows an additional control in which only the second antibody for mAb88B, anti-mouse IgG, was used in the immunoprecipitation. 25–100 μl of mouse hybridoma supernatant were used for the anti-Bip immunoprecipitations. As previously described (Blount and Merlie, 1988), ^{125}I-BTX-labeled BC3H-1 surface AChR was used in some experiments as an internal standard to calculate efficiencies of immunoprecipitations. For the reimmunoprecipitation of Bip-bound proteins, cells extracts were precipitated with Bip, the pellet was then resuspended in PBS, 0.1% Triton X-100, 10 mM MgCl, and 5 mM ATP, and incubated at room temperature for 45 min. The precipitate was repelleted in a microfuge, and the supernatant containing the proteins released by ATP treatment was reimmunoprecipitated. After immunoprecipitation (or reprecipitation), *Staphylococcus aureus* pellets were resuspended in sample buffer (containing 0.1% β-mercaptoethanol in the reduced gels) and subjected to SDS-PAGE on a 10% acrylamide, 0.27% *N,N'*-bis-methylene acrylamide

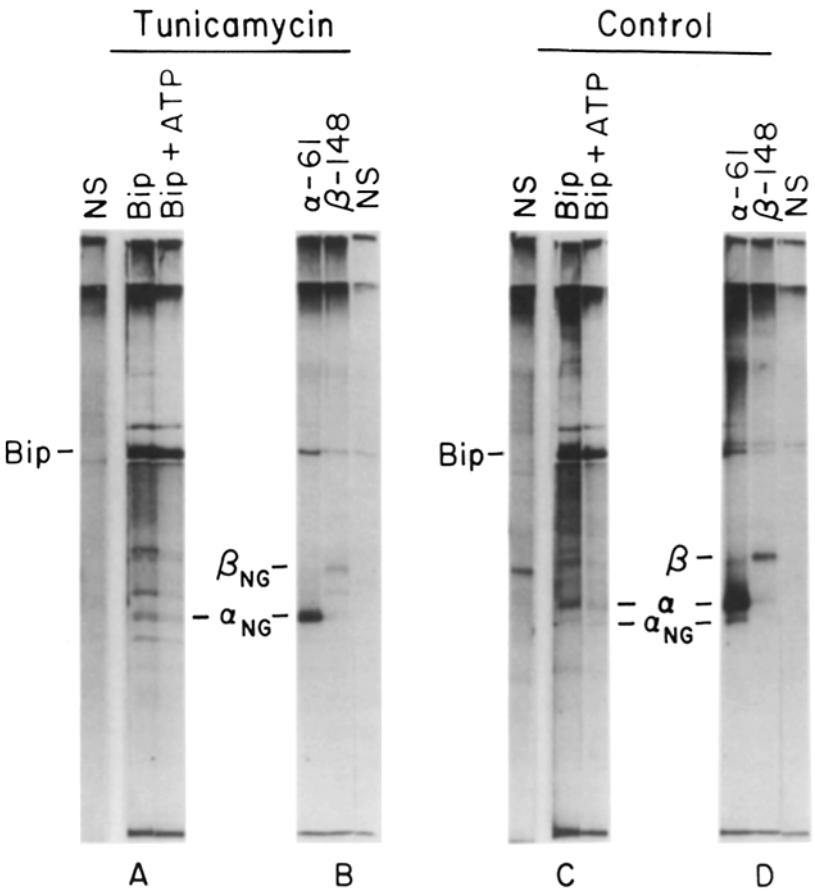

Figure 1. BC3H-1 AChR α and β subunits and subunit complexes are bound to Bip. BC3H-1 cells were incubated in the absence, *Control*, or presence of 1.5 μg/ml Tunicamycin for 10 h, pulse-labeled with [^{35}S]methionine for 7.5 min, harvested, extracted, and immunoprecipitated in *A* and *C* with anti-Bip antibody (*Bip* and *Bip + ATP*) or only the second antibody (*NS*) as described in Materials and Methods. The Bip precipitate was resuspended in PBS, 0.1% Triton X-100, 10 mM MgCl in the absence (*Bip*) or presence (*Bip + ATP*) of 5 mM ATP for 45 min at room temperature. The resulting supernatants from the ATP-treated precipitates were reimmunoprecipitated in *B* and *D* using the α specific mAb61 (*α-61*), the β-specific mAb148 (*β-148*), or only the second antibody (*NS*). All precipitates were analyzed by SDS-PAGE, and the fluorograms are shown. *A* and *C* were cut just below the Bip band so that two fluorographic exposure times could be shown; shorter fluorographic exposures are shown for Bip and larger molecular weight proteins, longer exposures are shown for low molecular weight proteins.

gel and buffer system (Laemmli, 1970). The gels were processed for fluorography using conditions such that band intensity was proportional to radioactivity and exposure time (Laskey and Mills, 1975). Quantitation was accomplished with a densitometer (LKB Instruments, Gaithersburg, MD).

Results

Bip Associates with AChR Subunits and Subunit Complexes

As an initial test of whether Bip bound AChR subunits, we stimulated Bip expression by tunicamycin treatment. Treated and untreated cultures of the muscle-like cell line, BC3H-1, were labeled briefly with [^{35}S]methionine and extracts were immunoprecipitated with an anti-Bip antibody. As seen in Fig. 1, even after extensive washing of the immunoprecipitate, many proteins were observed by SDS-PAGE and fluorography to coprecipitate with the Bip protein (Fig. 1, *A* and *C*, *Bip*). One such protein, not observed in overexposed fluorograms of nonspecific controls (NS), comigrated with the glycosylated and nonglycosylated α-subunit in control and tunicamycin-treated BC3H-1 cells respectively. To better assess whether AChR subunits were co-immunoprecipitated with Bip, we took advantage of the observation that Bip releases bound proteins in the presence of ATP and Mg^{2+} (Munro and Pelham, 1986). After ATP treatment, many Bip-associated proteins decreased in intensity, while the amount of Bip remained essentially unchanged (Fig. 1, *A* and *C*, *Bip* versus *Bip + ATP*). Subsequent reimmunoprecipitation of the ATP released supernatant with α- and β-subunit specific antibodies, mAb61 (*α-61*) and mAb148 (*β-148*) respectively, provided a sensitive assay for AChR subunits associated with Bip. Fig. 1, *B* and *D* demonstrates that Bip bound not only AChR subunits from tunicamycin-treated BC3H-1 cells, but also from untreated (*Control*) cells. Although the antibodies used were subunit specific, α and β subunits released from Bip by ATP treatment were observed to co-immunoprecipitate (note the presence of β in the α-61 lane and α in the β-148 lane) suggesting that some assembled, or partially assembled subunit complexes were also bound to Bip.

We have used a fibroblast expression system to better characterize Bip binding to α-subunit and subunit complexes. No qualitative difference has previously been observed between this expression system and AChR subunits expressed endogenously in BC3H-1 cells (Blount and Merlie, 1988, 1989; Blount et al., 1990; Phillips et al., 1991). Furthermore, the fibroblast expression system offers several advantages for the study of transient protein interactions: high level of expression of newly synthesized subunits, the ability to express selected subunits, and the potential to perform mutational analysis to study protein domains required for interaction. Q-α5,

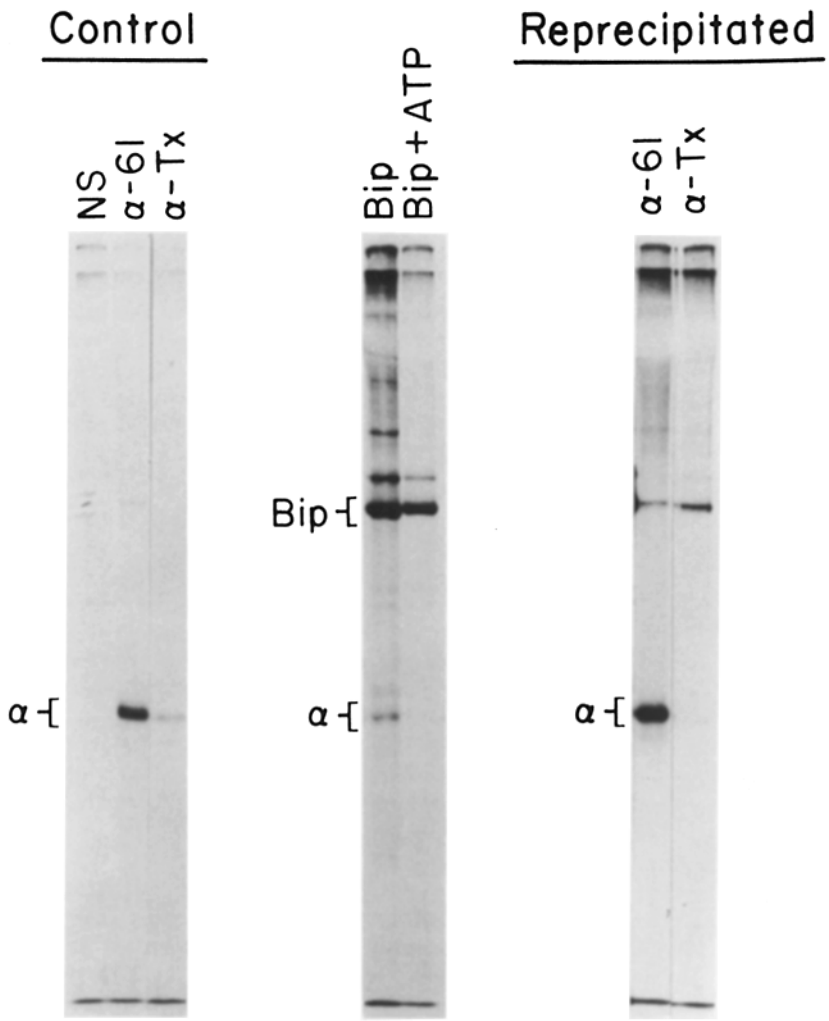

Figure 2. Bip associates preferentially with α_o rather than α_{Tx} subunit in transfected fibroblasts. Q-α5 cells were pulse-labeled for 1.5 min with [^{35}S]methionine, harvested, extracted, and immunoprecipitated as described in Materials and Methods. The resulting extract was immunoprecipitated with the α-subunit-specific mAb61 (*Control*, *α-61*), toxin antitoxin (*Control*, *α-Tx*), second antibody alone (*Control*, *NS*), or anti-Bip antibody. Because the incubation times with [^{35}S]methionine in the cell media were relatively short and steady-state labeling of all proteins was not achieved, no conclusions can be drawn from the quantity of Bip coimmunoprecipitating with the α-subunit. The Bip precipitate was subsequently incubated in the presence (*Bip + ATP*) or absence (*Bip*) of 5 mM ATP. The resulting supernatant from the ATP-treated precipitate was subsequently reprecipitated with mAb61 (*Reprecipitated*, *α-61*) and toxin antitoxin (*Reprecipitated*, *α-Tx*).

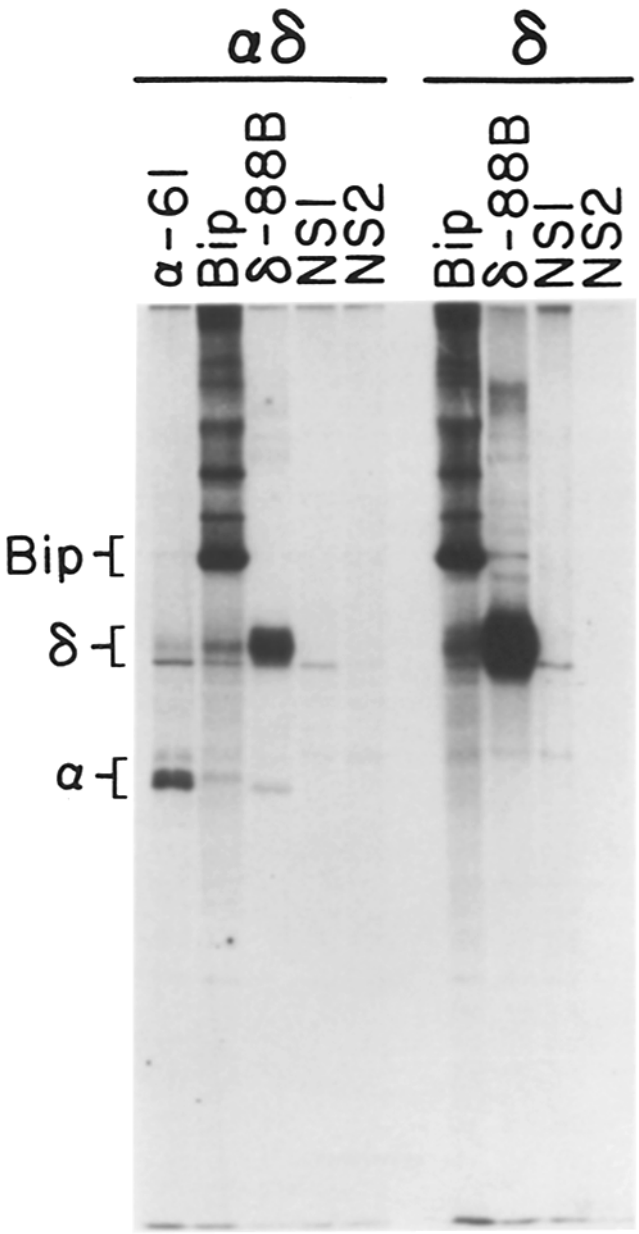

Figure 3. Proteins that comigrate with AChR α and δ subunits are associated with Bip in transfected fibroblasts. αδ and δ producing fibroblasts were pulse-labeled for 1 h with [^{35}S]methionine, harvested, extracted, and immunoprecipitated as described in Materials and Methods. The resulting extracts were immunoprecipitated with the δ-specific mAb88B (*δ-88B*), the anti-Bip antibody (*Bip*), or only the second antibody used for mAb61 and anti-Bip (*NSI*) or mAb88B (*NS2*). The mAb61 (*α-6I*) antibody was used to immunoprecipitate α-subunit from the αδ cell line.

a stably transfected fibroblast cell line expressing only the AChR α-subunit (Blount and Merlie, 1988), was examined for the presence of Bip/α-subunit complexes. As seen in Fig. 2, a protein with the electrophoretic mobility of the α-subunit coimmunoprecipitated with anti-Bip antibody (α in *Control* versus α in *Bip* lane). No α-like protein was co-precipitated in the parental fibroblast cell line, QT-6 (data not shown). ATP treatment of the Bip precipitate released the putative α-subunit without significantly decreasing the amount of Bip precipitated (Fig. 2, *Bip + ATP*). As previously observed for immunoglobulin heavy chain (Munro and Pelham, 1986), treatment with nonhydrolyzable ATP analogues did not significantly decrease the intensity of the putative α-subunit (not shown) suggesting that the release of AChR α-subunit from the precipitated pellet requires ATP hydrolysis. Reimmunoprecipitation of the ATP released supernatant with α-subunit-specific antibody mAb61 (*α-6I*) and with anti-toxin antibody (to precipitate α prebound with BTX, *α-Tx*) confirmed the identity of the α-subunit (Fig. 2, *Reprecipitated*).

Densitometric scanning of appropriate exposures of Fig. 2 revealed that α-subunit precipitated by toxin antitoxin was ~14% of the total α-subunit precipitated by mAb61 (*control*). However, <2.8% of the α-subunit precipitated with and released from Bip by ATP treatment could be reimmunoprecipitated by toxin antitoxin (*Reprecipitated*). Three additional independent experiments, two of which used ^{125}I-BTX-labeled AChR as an internal control to calculate immunoprecipitation efficiencies, confirmed that the ratio of α-subunit immunoprecipitated by BTX versus mAb61 was at least five-fold greater for total α than α-subunit bound to Bip and released by ATP treatment. Previous studies have demonstrated that mouse AChR α-subunit acquires the ability to bind BTX with high affinity in a time dependent manner (Merlie and Lindstrom, 1983; Blount and Merlie, 1988). Mutational analysis has indicated that formation of an intrachain disulfide bridge is required for this conformational maturation resulting in high-affinity BTX binding (Blount and Merlie, 1990). Hence, the data suggest that Bip preferentially associates with newly synthesized α-subunit, α_o, and associates less efficiently with the more mature α-subunit, α_{Tx}, that has acquired a disulfide bridge and high affinity for BTX.

Bip also bound α-subunit in stably transfected fibroblasts co-expressing α and γ or α and δ subunits. As shown in Fig. 3 (αδ), anti-Bip antibody (Bip) coprecipitated proteins that co-migrated with α (*α-6I*) and δ (*δ-88B*) subunits. Similar experiments performed on a cell line expressing only the δ-subunit suggested that unassembled δ also bound Bip (Fig. 3, δ). The identity of the δ subunit bound to Bip in this cell line expressing only unassembled δ subunit has been confirmed by reimmunoprecipitation of δ-subunit released from Bip by ATP treatment (not shown). Similarly, the identities of the α and δ subunits in the αδ cell line were confirmed by reimmunoprecipitation of proteins released from Bip by ATP treatment (Fig. 4, *Reprecipitated*). Although no Bip associated α_{Tx} subunit was detected, a small amount of coprecipitation of α and δ subunits suggested that Bip bound not only unassembled subunits, but also αδ subunit complexes. Similarly, reimmunoprecipitation of proteins released by ATP treatment from a fibroblast cell line stably transfected with and expressing α and γ subunits demonstrated that α_o, not α_{Tx}, was associated with Bip in an ATP releasable manner (Fig. 5 *A*, *Re-ppt*). Upon long exposure, αγ complexes were observed (Fig. 5 *B*, *Reprecipitated*). Thus, these data suggest that Bip binds the α_o subunit and a small amount αδ and αγ complexes containing the newly translated α_o subunit, but does not efficiently bind the disulfide bridged and conformationally mature α_{Tx} subunit.

Electrophoretic Mobility of the Newly Synthesized α_o and Conformationally Mature α_{Tx} Subunit

As shown in Figs. 3 and 5, α-subunit bound to Bip had

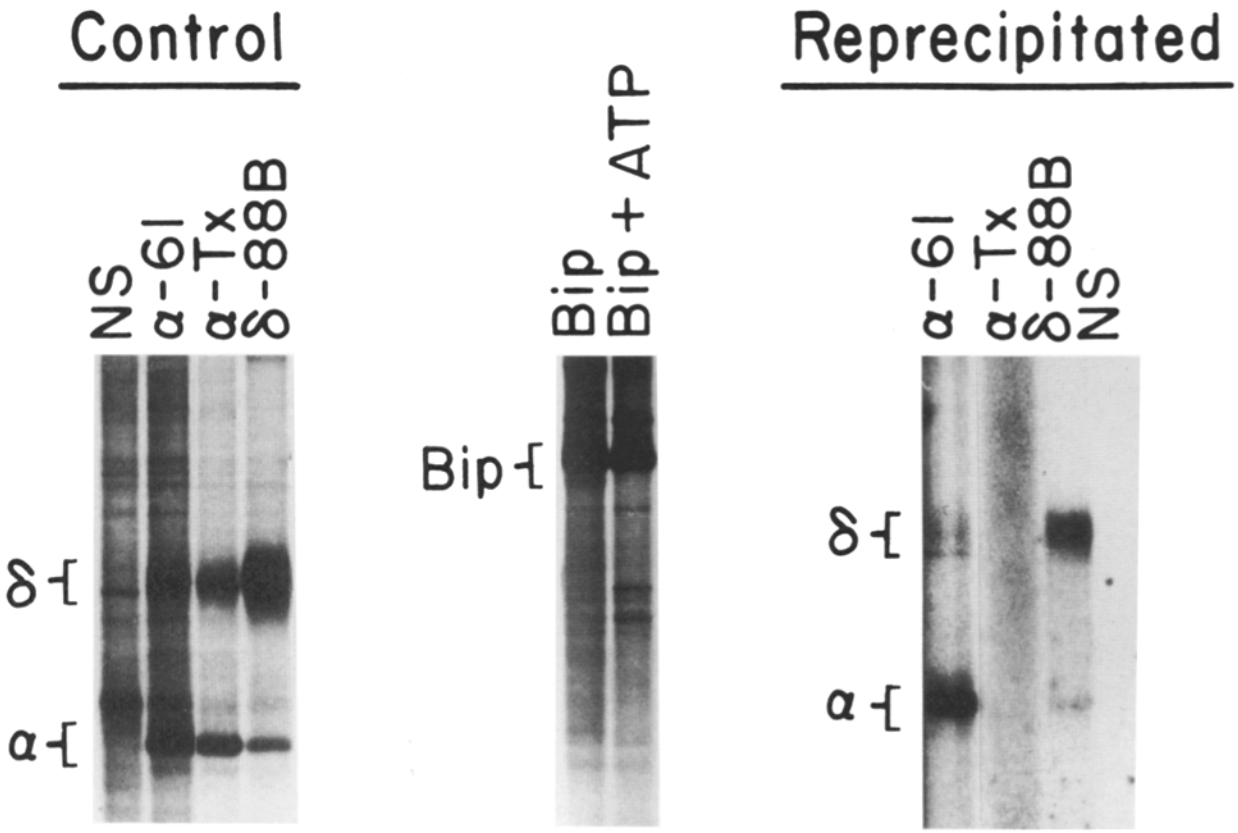

Figure 4. AChR α and δ subunits and αδ complexes are associated with Bip. The αδ cell line was pulse-labeled for 4 h with [^{35}S]methionine, harvested, extracted, and immunoprecipitated as described in Materials and Methods. The resulting extracts were immunoprecipitated with the α-specific mAb61 (*Control, α-61*), toxin anti-toxin (*Control, α-Tx*), the δ-specific mAb88B (*Control, δ-88B*), only second antibody (*Control, NS*), or the anti-Bip antibody. The anti-Bip precipitate was subsequently incubated in the presence (*Bip + ATP*) or absence (*Bip*) of 5 mM ATP. The resulting supernatant from the ATP-treated precipitate was then reprecipitated with mAb61 (*Reprecipitated, α-61*), toxin anti-toxin (*Reprecipitated, α-Tx*), mAb88B (*Reprecipitated, δ-88B*), or only the second antibody (*Reprecipitated, NS*).

slightly less electrophoretic mobility in SDS-PAGE than did α_{Tx} or the α-subunit in αδ complexes. This difference in electrophoretic mobility was observed only when samples were not reduced; no difference in migration was observed when 0.1% β-mercaptoethanol was included in the SDS sample buffer (not shown). Consistent with the hypothesis that the electrophoretic mobility of α-subunit was influenced by disulfide bridging of cysteines at positions 128 and 142, we observed the predicted difference in mobility when a mutant α-subunit expressed in two independent fibroblast clones, containing serines rather than cysteines at these positions (Blount and Merlie, 1990), was compared with wild-type α_{Tx} (Fig. 6). As demonstrated in Fig. 6, *top*, mutant and wild-type subunits analyzed by SDS-PAGE under nonreduced conditions (*Not Reduced*) had different electrophoretic mobilities similar to the difference observed between α associated with Bip and α_{Tx}. No difference in migration was observed when the same samples were treated with a reducing agent (*Reduced*). Similarly, as demonstrated in Fig. 6, *bottom*, the wild-type α-subunit that assembled with δ had an electrophoretic mobility identical to the faster migrating α_{Tx} species. By contrast, mutant α-subunit associated with δ maintained the lesser mobility (*Not Reduced*). This difference in mobility was similarly sensitive to reducing agents (*Reduced*). Taken together, these data suggest that Bip associates preferentially with the newly synthesized α_o subunit and αγ and αδ complexes containing α_o, Bip binds less efficiently to the α_{Tx} subunit which, because of a disulfide bridge between cysteines located at amino acid positions 128 and 142, has a higher affinity for BTX and a greater electrophoretic mobility in SDS-PAGE.

Discussion

Here we have shown that α_o and heteromeric complexes of $\alpha_o\delta$ and $\alpha_o\gamma$, but not the conformationally mature α_{Tx} and heteromers containing α_{Tx}, bind Bip. Previous studies have demonstrated that Bip binds to immunoglobulin heavy chain before assembly (Haas and Wabl, 1983; Bole et al., 1986; Hendershot, 1990), to influenza virus hemagglutinin (Hurtley et al., 1989) and to simian virus 5 type II hemagglutinin-neuraminidase (Ng et al., 1989) before protein folding, and to vesicular stomatitis virus G protein before disulfide bond formation (Machamer et al., 1990). These observations led to the speculation that Bip plays an important role as a molecular chaperon in the biosynthesis of some multimeric proteins. However, whether Bip functions to aid assembly (Haas and Wabl, 1983), retain newly synthesized proteins in the ER (as might be suggested by Bip's KDEL ER retention sequence; Munro and Pelham, 1987), target misfolded proteins for degradation, prevent aggregation of ER proteins during times of stress (Munro and Pelham, 1987), or assist in the

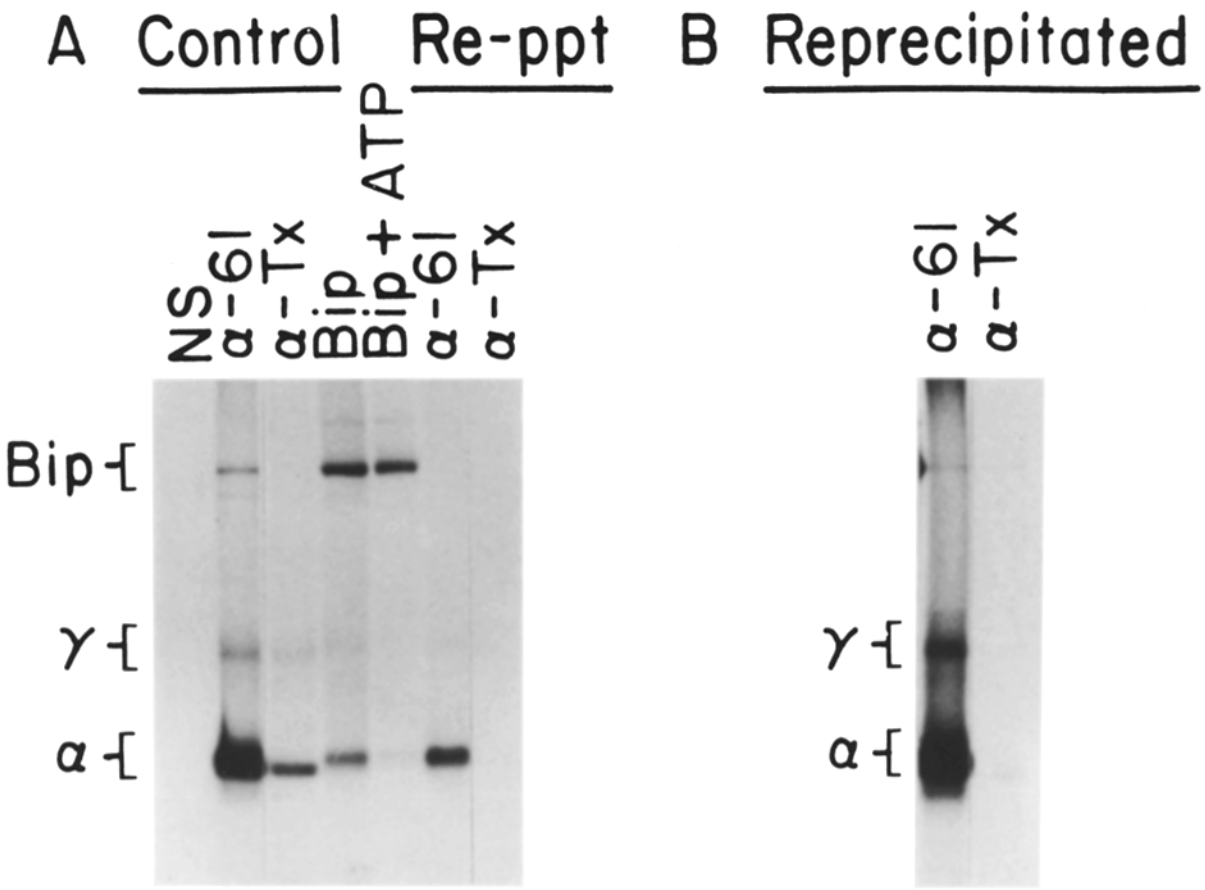

Figure 5. AChR α-subunit and αγ complexes are associated with Bip. The αγ cell line was pulse-labeled for 4 h with [^{35}S]methionine, harvested, extracted, and immunoprecipitated as described in Materials and Methods. The resulting extracts were immunoprecipitated with the α specific mAb61 (*Control*, *α-61*), toxin anti-toxin (*Control*, *α-Tx*), only the second antibody (*Control*, *NS*), or the anti-Bip antibody. Because the incubation times with [^{35}S]methionine in the cell media were relatively short and steady state labeling of all proteins was not achieved, no conclusions can be drawn from the quantity of Bip coimmunoprecipitating with the α-subunit. The Bip precipitate was subsequently incubated in the presence (*Bip + ATP*) or absence (*Bip*) of 5 mM ATP. The resulting supernatant from the ATP treated precipitate was then reprecipitated with mAb61 (*Re-ppt*, *α-61*), toxin anti-toxin (*Re-ppt*, *α-Tx*). *B* shows a longer exposure of the Re-ppt lanes shown in *A*.

folding of newly synthesized subunits (Ng et al., 1989; Machamer et al., 1990) remains unclear. Our work on the processing and assembly of the AChR subunits (for review, see Blount and Merlie, 1991) suggested some roles for Bip in AChR biogenesis.

A previous study demonstrated that the α-subunit acquires high-affinity BTX binding in a time-dependent manner independent of assembly with other subunits (Merlie and Lindstrom, 1983; Blount and Merlie, 1988). Expression of mutated α-subunits in a fibroblast expression system demonstrated that this change in α-subunit conformation is dependent upon two cysteines at position 128 and 142 that are known to form a disulfide bond (Blount and Merlie, 1990). Hence, the evidence strongly supports the hypothesis that disulfide bridging of these two cysteines is the time-dependent covalent modification required for the formation of a high-affinity binding site for BTX. Subcellular fractionation of BC3H-1 cells demonstrated that conformational maturation and subunit assembly occur in the ER (Smith et al., 1987). Consistent with this result, characterization of αδ and αγ subunit complexes expressed in fibroblasts suggested that assembly intermediates were formed in and confined to a pre-Golgi compartment (Blount et al., 1990). Hence, any cellular machinery that may be required for AChR subunit conformational maturation or assembly is likely to reside within the ER.

The observation that Bip, a resident ER protein, binds preferentially to the conformationally immature α_o subunit suggests a limited number of roles Bip may play in AChR biogenesis. Because not all forms of unassembled α-subunit are bound to Bip, it seems unlikely that Bip is responsible for the retention of unassembled and incompletely assembled subunits in the ER (Smith et al., 1987; Blount and Merlie, 1988; Blount et al., 1990). In addition, because there is no correlation between subunits being released from Bip and the formation of αδ or αγ heteromers, Bip is unlikely to play a role in AChR subunit assembly. Finally, although there exists a correlation between the rapid degradation of the $\alpha_o\delta$ complex (Blount and Merlie, 1990) and its association with Bip, we have previously demonstrated that unassembled α_o has a degradation rate indistinguishable from that of the α_{Tx} subunit (Blount and Merlie, 1988); therefore, it seems unlikely that Bip targets immature subunits for rapid degradation. Our demonstration that the release of Bip from α_o

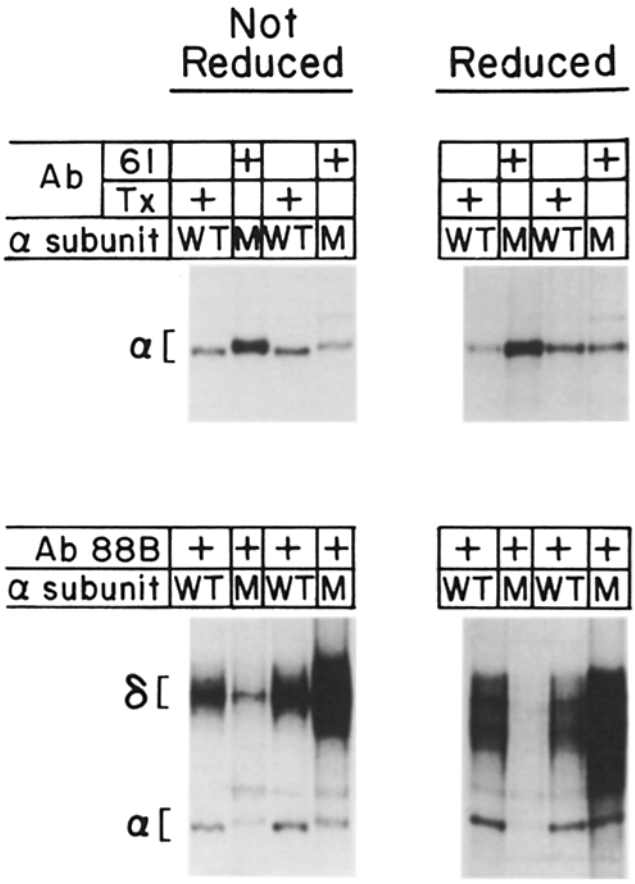

Figure 6. Nonreduced mutant α_o and wild-type α_{Tx} showed a difference in electrophoretic mobility. Cells from a single $\alpha\delta$ cell line and two independent cell lines coexpressing the 128C → S + 142C → S mutant α and wild-type δ subunits (Blount and Merlie, 1990) were pulse-labeled with [^{35}S]methionine for 2 h, harvested, extracted, and immunoprecipitated as described in Materials and Methods. Extracts from all cell lines were immunoprecipitated using the δ specific mAb88B (*Ab 88B*) to immunoprecipitate α-subunit associated with δ. Toxin antitoxin (*Ab TX*) and mAb61 (*Ab 61*) were used to immunoprecipitate the α_{Tx} and the mutant α_o from the wild-type and mutated α-subunit producing cell lines, respectively. All precipitates were resuspended in running buffer (*Not Reduced*), and to half, 0.1% of β-mercaptoethanol was added, and the suspension was placed in a boiling water bath for 5 min before analysis (*Reduced*). All samples were subsequently analyzed by SDS-PAGE with the wild-type (*WT*) and mutant (*M*) α subunits in alternating lanes. The resulting fluorograms are shown.

correlates with formation of the 128–142 disulfide bond and transition to the mature α_{Tx} conformation suggests that more likely roles for Bip are: (*a*) Bip prevents aggregation of immature subunits, or, (*b*) Bip assists in the folding of immature subunits, thus allowing or facilitating the formation of an intramolecular disulfide bridge. As a corollary, Bip must dissociate upon disulfide bond formation or conformational maturation. Future studies using site-directed mutagenesis to determine domains required for AChR subunit–subunit and subunit–Bip associations, analysis of the kinetics of these interactions, and the development of a cell-free translation system capable of synthesizing assembled AChR may yield additional clues to the functional role Bip plays in the biogenesis of the muscle AChR.

We would like to acknowledge Dr. Linda Hendershot for the anti-Bip mAb and for originally suggesting that AChR subunits in tunicamycin treated BC3H-1 cells may bind Bip. We also would like to thank Drs. Stanley Froehner and Jon Lindstrom for the use of their AChR antibodies. Dr. McHardy Smith for helpful discussion, and Despina Ghement for technical assistance in growth of the BC3H-1 cell line.

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