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 multi-subunit 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 subunit 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 α3, and α6 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), 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 2α2βγδ (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 αδ complexes containing conformationally immature α-subunit were degraded more rapidly than αδ 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-
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 αγ and αδ 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 α2-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); [35S]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 (Moscovici 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 β subunits (Blount and Merlie, 1989) of the mouse AChR have been described. Pulse labeling with [35S]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 α2 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), 125I-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 mouse hybridoma supernatant, and pelleted by centrifugation. 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...
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 [35S]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 Mg2+ (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](image-url)

**Figure 2.** Bip associates preferentially with αo rather than α7 subunit in transfected fibroblasts. Q-α5 cells were pulse-labeled for 1.5 min with [35S]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 [35S]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 precipitated with mAb61 (Reprecipitated, α-61) and toxin antitoxin (Reprecipitated, α-Tx).
Proteins that comigrate with AChR α and δ subunits are associated with Bip in transfected fibroblasts, αδ producing fibroblasts were pulse-labeled for 1 h with [35S]methionine, harvested, extracted, and immunoprecipitated as described in Materials and Methods. The resulting extracts were immunoprecipitated with the δ-specific mAb 88B (δ-88B), the anti-Bip antibody (Bip), or only the second antibody used for mAb 61 and anti-Bip (NS1) or mAb 88B (NS2). The mAb 61 (or-61) antibody was used to immunoprecipitate α-subunit from the αδ cell line.

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 [35S]methionine, harvested, extracted, and immunoprecipitated as described in Materials and Methods. The resulting extracts were immunoprecipitated with the δ-specific mAb 88B (δ-88B), the anti-Bip antibody (Bip), or only the second antibody used for mAb 61 and anti-Bip (NS1) or mAb 88B (NS2). The mAb 61 (or-61) antibody was used to immunoprecipitate α-subunit from the αδ cell line.

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AChR α and δ subunits and αδ complexes are associated with Bip. The αδ cell line was pulse-labeled for 4 h with [35S]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 αγ 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 αγ, (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 αγ. 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 αγ 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, subunit and αγ and αδ complexes containing αγ, Bip binds less efficiently to the αγ 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, and heteromeric complexes of αδ and αγ, but not the conformationally mature αγ, and heteromers containing αγ, 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
AChR α-subunit and γ2 complexes are associated with Bip. The γ2 cell line was pulse-labeled for 4 h with [35S]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 [35S]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.

The observation that Bip, a resident ER protein, binds preferentially to the conformationally immature α, 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 α,δ complex (Blount and Merlie, 1990) and its association with Bip, we have previously demonstrated that unassembled α, has a degradation rate indistinguishable from that of the α,γ 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,
Figure 6. Nonreduced mutant αc and wild-type αct showed a difference in electrophoretic mobility. Cells from a single αct 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 [35S]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 also used to immunoprecipitate the αc, and the mutant αc 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 αct, conformation suggests that more likely roles for Bip are: (a) Bip prevents aggregation of immature subunits, or (b) Bip assists in the folding 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.

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