Involvement of the Chaperone Protein Calnexin and the Acetylcholine Receptor β-Subunit in the Assembly and Cell Surface Expression of the Receptor*

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The nicotinic acetylcholine receptor at the neuromuscular junction is a ligand-gated ion channel assembled in the endoplasmic reticulum from four distinct glycoprotein subunits into the pentameric configuration of αßγδ. The individual homologous subunits form specific contacts at interfaces with neighboring subunits to achieve the appropriate orientation and order of each subunit in surrounding the ion channel. Assembly is thought to proceed through the formation of intermediates composed of dimers of the αδ and αγ subunits which are eventually joined by the β-subunit to achieve a circular structure enclosing the gated ion channel. In this study, we transfected cDNAs encoding receptor subunits in various combinations into HEK-293 cells to identify intracellular factors that influence the assembly and cell surface expression of the receptor. Our data derived from brefeldin A-treated cells indicate that intracellular association of the receptor subunits with the β-subunit increases the pool of fully assembled receptors available for transport to the cell surface, presumably by protection from degradation. In addition, we determined that the chaperone protein calnexin is associated with the isolated α-, β-, and δ-subunits of the receptor, but calnexin is not detected in association with assembled αδ subunit dimers. Calnexin is also detected in association with maturely folded, unassembled α-subunits, as observed by the recognition of this complex by the monoclonal antibody mAb 35, believed to be specific for correctly folded α-subunits. Thus, calnexin appears to associate with the individual nascent subunits, thereby facilitating their assembly into the mature pentameric receptor.

Subunits of the nicotinic acetylcholine receptor at the neuromuscular junction are assembled into a pentagonal shaped structure positioned around a central ion channel pore, a structure shared by other ligand-gated ion channels (Noda et al., 1983; Smith et al., 1987; Unwin, 1989; Machold et al., 1995). All five subunits form contacts at two opposing interfaces; the subunit contact points should occur at corresponding positions in their homologous sequences (Kreienkamp et al., 1995). The homologous subunits are assembled in the endoplasmic reticulum with a circular arrangement of α-γ-α-δ-β (Karlin and Akabas, 1995; Machold et al., 1995). Complete assembly with the β-subunit, the last subunit thought to join the assembly sequence (Saedi et al., 1991; Sine and Claudio, 1991; Gu et al., 1991a; Kreienkamp et al., 1995), is a requirement for this receptor to be expressed at the cell surface, escaping retention in the endoplasmic reticulum where unassembled subunits have a higher propensity of being degraded (Merlie and Lindstrom, 1983; Smith et al., 1987; Blount and Merlie, 1990). Previous studies have investigated factors which influence subunit assembly and expression of the receptor at the cell surface and have identified amino acids responsible for the contact interfaces between subunits (Yu et al., 1991; Gu et al., 1991b; Verrall and Hall, 1992; Kreienkamp et al., 1995), stabilization of assembled subunits protecting them from degradation (Blount and Merlie, 1988; Blount et al., 1990), glycosylation (Smith et al., 1986; Sumikawa and Miledi, 1989; Buller and White, 1990; Gehle and Sumikawa, 1991), and association with chaperone proteins (Paulsen et al., 1991; Blount and Merlie, 1991; Forsayeth et al., 1992; Gelman et al., 1995).

Several cellular processes involving chaperone proteins might be hypothesized as being involved in the assembly of the acetylcholine receptor. A low affinity association of chaperone proteins to unassembled receptor subunits may inhibit aggregation and preclude the formation of spurious contacts with other proteins localized to the endoplasmic reticulum (ER) membrane by overlaying areas where nonspecific contacts are likely to occur (mechanism reviewed in Hendrick and Hartl (1993)). Large chaperone proteins that overlay unassembled subunit precursors may provide protection from degradation in the ER. Chaperone proteins, with endoplasmic reticulum retention codes, that are bound to unassembled receptor subunits may prevent premature export of unassembled subunits to the cell surface (Hendershot et al., 1987; Rajagopalan et al., 1994; Bergeron et al., 1994). The adherence of abundant chaperone proteins to receptor subunits that are easily displaced when contacts of higher affinity are formed between neighboring receptor subunits may selectively guide the assembly pathway.

By using transient expression of mouse receptor subunits in HEK-293 cells, which do not naturally express the acetylcholine receptor, we demonstrate that association of the β-subunit with the other assembly intermediates of the receptor increases the intracellular pool of folded receptor protein. In addition, we demonstrate that the chaperone protein calnexin is associated with unassembled β-δ- as well as α-subunits of the receptor, but is not detected in association with assembled subunit dimers and assembled receptor pentamers. Previous studies on the chaperones Bip (Blount and Merlie, 1991; Forsayeth et al., 1994)

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1992) and calnexin (Gelman et al., 1995) indicate that they adhere to unassembled α-subunits of the acetylcholine receptor in COS cells and embryonic chicken muscle cells. Calnexin is an abundant 90-kDa protein, which is approximately twice the size of an acetylcholine receptor subunit, transverses the ER membrane, and possesses an ER retention signal in its cytoplasmic domain (Bergeron et al., 1994; Ou et al., 1995). These characteristics give calnexin an opportunity to overlay unassembled receptor subunits and retain them in the ER.

Our data also indicate that calnexin is associated with folded receptor subunits, indicating that its role extends beyond binding to nascent polypeptide chains and contributing to their folding pathway. The adherence of this chaperone protein to unassembled receptor subunits and its detectable absence in association with more highly assembled intermediates indicate that calnexin has a role in the assembly pathway of the acetylcholine receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**

$^{125}$I-α-Bungarotoxin (specific activity of 1.1 × 10$^5$ Ci/mmol) was obtained from DuPont NEN. Monoclonal antibodies directed to the α-subunit (mAbs 35 and 210), β-subunit (mAbs 111, 118, and 124), and δ-subunit (mAb 137) were used in the immunoprecipitations and Western blots as indicated. A δ-subunit antibody mAb 88B was a gift of Dr. Dr. Staudt at the University of North Carolina. The calnexin antibody directed to the carboxyl terminus of calnexin used for immunoprecipitation and Western blots was purchased from Stressgen (Victoria, British Columbia, Canada). Peroxidase-conjugated anti-mouse antibody was purchased from Life Technologies (Gaithersburg, MD), the peroxidase-conjugated anti-rabbit antibody to detect the calnexin antibody on Western blots was purchased from Amersham and the peroxidase-conjugated anti-β-gal antibody used as the secondary antibody to detect the δ-subunit specific primary antibodies was purchased from Jackson Laboratories (Westgrove, PA). The ECL chemiluminescence detection system was from Amersham, the immunoprecipitation beads (UltraLink Protein-G resin) and the Amino-Link Kit to conjugate mAbs 137 and 88B for the immunoaffinity isolation of acetylcholine receptors from C2 cells were purchased from Pierce. Brefeldin A, carboxymethylcholine, and all other reagents were purchased from Sigma. Pre-cast gels were purchased from Novex (San Diego, CA).

**Expression of the Receptors—**  

The receptor subunits were transfected by calcium phosphate precipitation and expressed in HEK cells as indicated by Keller et al. (1995). α-Subunit cDNA (15 μg) and 7.5 μg of β- and δ-subunit cDNA, or an equal volume mixture of mAbs 111, 118, and 124 for the α-subunit, and an equal volume mixture of mAbs 88B and 137 for the δ-subunit, were co-transfected in each 10-cm culture dish, except for the co-transfection of α and δ and α and β, where 15 μg of δ-subunit cDNA was transfected. Acetylcholine receptors were also studied in differentiated mouse muscle cells from the cell culture line C2C12 (from American Type Culture Collection). For receptors expressed in brefeldin A, cells were incubated for 1 h prior to the transfection in 7 mM brefeldin A, and the same concentration was maintained in the media until the cells were harvested.

**Binding Assays—**  

Cells were harvested in phosphate-buffered saline with 1 mM CaCl$_2$ to measure receptors at the cell surface, the cells were resuspended in K$^-$-Ringers buffer, an aliquot was incubated in 10 mM carboxymethylcholine, and all cells were then incubated with 20 mM $^{125}$I-α-Bgtx for approximately 3 h. Unbound toxin was removed by washing three times in a large volume of K$^-$-Ringers buffer, cells were sedimented and bound $^{125}$I-α-Bgtx was measured in a γ-counter. To measure the total cellular expression of the receptor, that is the intracellular and the cell surface expression of the receptor, cells were permeabilized in a saponin buffer (0.1% saponin, 10 mM EDTA, 0.1% bovine serum albumin, 10 mM NaPO$_4$, pH 7.4), which allows $^{125}$I-α-Bgtx to access the receptor at intracellular and extracellular locations. Expression of α-subunits in cells transfected with α alone was estimated by subtracting the amount of $^{125}$I-α-Bgtx binding to untransfected cells from the amount of $^{125}$I-α-Bgtx binding to an equivalent population of transfected cells. To quantify unassembled α-subunits in cells co-transfected with α and δ, the amount of $^{125}$I-α-Bgtx binding to untransfected cells was subtracted from the amount of $^{125}$I-α-Bgtx binding to the detergent extraction buffer, and bound material was eluted in 0.1 M glycine buffer, pH 2.5. Afterwards, the pH was adjusted to approximately pH 7.0 using 1 M Tris, pH 9.0, samples were concentrated in a Centricon concentrator and then mixed in Laemmli sample buffer at a dilution ratio of sample to 1:10. Western blots were developed according to the ECL protocol (Amersham), using the appropriate secondary antibody.

**RESULTS**

**Intracellular Subunit Assembly and Expression—** To quantify receptor expression and assess the role of assembly with the β-subunit to receptor expression intracellularly and at the cell surface, binding assays were performed with the slowly dissociating antagonist $^{125}$I-α-Bungarotoxin (Bgtx). Prior incubation of cells with carboxymethylcholine inhibits α-Bgtx binding at the αδ and αγ interfaces but does not prevent α-toxin recognition of unassembled α-subunits (Blount and Merlie, 1989; Sine and Claudio, 1991), providing a means to quantify the specific binding of α-Bgtx to αδ and αγ dimers and to distinguish unassembled α-subunits from those associated with the δ- or γ-subunits. When cDNAs encoding α-, γ-, and δ-subunits are co-transfected, unassembled α-subunits, αδ and αγ dimers, and the tetramer αγγδ are detected at the intracellular locations in HEK-293 cells (Kreienkamp et al., 1995). Inclusion of the β-subunit in a transfection leads to the biogenesis of the fully assembled receptor pentamer on the cell surface in the configuration αδβγδ, as well as the intracellular presence of pentamers, dimers, and monomers (Kreienkamp et al., 1995). Cells co-transfected with α, β, and δ express a receptor pentamer αδβγδ at the cell surface, although the expression levels are substantially lower than in the natural configuration of αδβγδ (Sine and Claudio, 1991). The inclusion of the β-subunit increases cell surface expression approximately 9-fold in the co-transfection of α, β, and δ (Fig. 1A) and approximately
10-fold upon co-transfection with α, β, δ, and γ (Fig. 1B).

All values shown in Fig. 1 are standardized by subtracting the amount of 125I-α-Bgtx binding to cells incubated with 10 mM carbamoylcholine.

Cells in culture were treated with brefeldin A to measure the contribution of association of the β-subunit with receptors localized in the intracellular compartments of the cell. Brefeldin A collapses the Golgi apparatus into the endoplasmic reticulum compartment and results in the elimination of the cellular machinery that exports proteins to the cell surface (MIsumi et al., 1986; Lippincott-Schwartz et al., 1989; Klausner et al., 1992). Treatment with 7 nM brefeldin A for the transfection interval of approximately 50 h virtually eliminates expression of the receptor at the cell surface (Fig. 2A). Although the total intracellular protein expression is also reduced in 7 nM brefeldin A, detectable expression of receptor protein persists. The addition of the β-subunit in the transfection increases by approximately 3-fold the number of intracellular 125I-α-Bgtx binding sites protected by carbamoylcholine (Fig. 2B). Since the number of carbamoylcholine protected 125I-α-Bgtx binding sites is indicative of the amount of α-subunits associated with δ- and γ-subunits, co-transfection of the β-subunit also increases the intracellular concentration of α-, δ-, and γ-subunit protein by approximately a factor of three.

Co-immunoprecipitation and Western blot analysis of the receptor subunits expressed in brefeldin A were used as an additional means to estimate the levels of receptor expression and to demonstrate that the β-subunit associates with the δ- and α-subunits in brefeldin A-treated cells. (Suitable antibody was not available to conduct parallel experiments with the γ-subunit.) Detergent extracts of transfected cells were immunoprecipitated with mAb 35, an antibody recognizing α-subunits in their native conformation (Tzartos et al., 1981) and receptor subunits were monitored on Western blots. The banding densities of the α-subunit and δ-subunit in the Western blots significantly increase upon co-transfection with α, β, γ, and δ (α-subunit, Fig. 3A, lane 2; α- and δ-subunits, Fig. 3B, lane 4) relative to α, δ, and γ (α-subunit, Fig. 3A, lane 1; α- and δ-subunits, Fig. 3B, lane 3), indicating that co-expression of the β-subunit also increases the expression or stability of the other receptor subunits. To demonstrate that the β-subunit is associated with the α- and δ-subunits in the co-transfection of α, β, γ, and δ, the immunoprecipitated sample was resolved in gels and probed in the Western blot with monoclonal antibodies that recognize the β-subunit (Fig. 3C, lane 5). The Western blot showing the presence of the β-subunit in Fig. 3C, lane 5, demonstrates that the β-subunit associates with the α- and δ-subunits in brefeldin A-treated cells.

To further demonstrate that increases in the presence of the α- and δ-subunits are due to association with the β-subunit and also to assess the variability in receptor expression levels among several transfections, 125I-α-Bgtx binding was performed in cells transfected with α-subunit cDNA alone and co-transfected with α- and β-subunit cDNA (Fig. 2C). In each case, three separate transfections were used. The β-subunit does not appear to associate with the α-subunit in the absence of the δ- or γ-subunits, as based on the observations that when cells are co-transfected with α and β, immunoprecipitated with the α-subunit specific antibody mAb 35 and the Western blot is developed with antibodies specific to both α- and β, only the α-subunit is detected on the Western blot. However, the β-subunit is detected in the same Western blot upon co-transfection with α, β, γ, and δ and a similar immunoprecipitation (data not shown). Similar to our observations, Gu et al. (1991a) observed that the α-subunit does not associate with β-subunit alone. Our data demonstrate similar expression levels between the transfection of only α and the co-transfection of α and β (Fig. 2C). This contrasts with data where the α-, β-, γ-, and δ-subunits are co-transfected, there an elevated accumulation of receptor subunits associated with the β-subunit are observed.
These combinations of receptor subunits are believed to form alignments with migration distances of the receptor subunits bodies directed to the detected subunit, their absence of reactivity to the anti-α, β, γ, and δ and detergent extracts were immunoprecipitated with mAb 35. After electrophoresis of the immunoprecipitated samples, proteins on the Western blots were detected with specific antibodies to the α-, β-, γ-, and δ-subunits. The locations of the receptor subunits and the mAb 35 IgG heavy chain present in the immunoprecipitation mixture are indicated on the blot (IP: immunoprecipitation). A, Western blot was probed with mAb 210 which recognizes the α-subunit. Cells were co-transfected with α, γ, and δ (lane 1) and α, β, γ, and δ (lane 2), B, the same samples as in A, co-transfected with α, γ, and δ (lane 3) and α, β, γ, and δ (lane 4) and probed in the Western blot with a mixture of antibodies that identify the δ-subunit (mAbs 88B and 137) and the α-subunit (mAb 210). C, the same Western blot lane shown in panel A, lane 2 (cells transfected with α, β, γ, and δ), re-probed with antibodies to the β-subunit (mAbs 111, 118, and 124), demonstrating association of the β-subunit to the receptor in brefeldin A-treated cells. The fainter appearance of the protein band identified as the β-subunit compared to the α- and δ-subunit bands in the other panels, and the absence of the IgG band are presumably due to this blot being re-probed several times.

and 3). These data also indicate that the variability of expression levels found among several parallel transfections (Fig. 2C) is significantly less than the increase of receptor subunit protein observed when the β-subunit associates with the receptor (Figs. 2B and 3).

The Chaperone Protein Calnexin Is Associated with Unassembled α-, β- and δ-Subunits in Transfected HEK Cells.—To determine whether calnexin is associated with various combinations of receptor subunits, an antibody to calnexin was used to co-immunoprecipitate receptor subunits and samples were resolved on Western blots with antibodies that recognize receptor subunits. Cells were transfected with only α, δ, or β, co-transfected with α and δ which assemble into αδ dimers (Kreienkamp et al., 1995), co-transfected with α, γ, and δ which forms the dimers αδ, aγ, and a tetramer aγaγ (Kreienkamp et al., 1995) and co-transfected with α, β, γ, and δ which results in the expression of the αδ and αγ dimer intermediates intracellularly and the expression of receptor (α,βγδ) pentamer intracellularly and at the cell surface (Kreienkamp et al., 1995). These combinations of receptor subunits are believed to form intermediates in the assembly pathway leading toward the biogenesis of the mature receptor pentamer α2βγδ (Kreienkamp et al., 1995). The ratios and amounts of cDNA coding for each subunit were similar in each transfection as were the number of plates of cells transfected and later solubilized in detergent. The protein bands observed on Western blots were identified as receptor subunits by their reactivity to the antibodies directed to the detected subunit, their absence of reactiivity to antibodies directed to related subunits and by their alignments with migration distances of the receptor subunits expressed endogenously in the mouse muscle cell line C2C12 (α-subunit, Fig. 4A, lane 1; α- and β-subunits, Fig. 4B, lane 1; δ-subunit, Fig. 4C, lane 1). The methods utilized in this study enabled us to identify clearly the α-, β-, and δ-subunits.

The calnexin co-immunoprecipitation experiments indicate calnexin is associated with unassembled α-, β-, and δ-subunits, since dense protein bands corresponding to the receptor subunits derived from cells transfected with isolated receptor subunits are observed in Western blots (α-subunit, Fig. 4A, lane 2; β-subunit, Fig. 4B, lane 3; δ-subunit, Fig. 4C, lane 3). The α- and δ-subunits bands obtained from the calnexin co-immunoprecipitation in cells co-transfected with α, β, γ, and δ (α-subunit, Fig. 4A, lane 5; δ-subunit, Fig. 4C, lane 5) are fainter than those found in their respective single subunit transfections.
tions (α-subunit, Fig. 4A, lane 2; δ-subunit, Fig. 4C, lane 3). In addition, the β-subunit is not appreciably detected in the co-immunoprecipitation when cells are co-transfected with α, β, γ, and δ (Fig. 4B, lane 5), although the β-subunit is detected in Western blots derived from these cellular extracts prior to immunoprecipitation (data not shown). Our co-immunoprecipitations in cells transfected with α, β, γ, and δ should detect the receptor in the intracellular and extracellular locations of the cell, because it has been demonstrated that significant amounts of fully assembled receptor pentamers are located at the cell surface, because co-immunoprecipitation did not yield the entire receptor complex with calnexin.

The ratios of subunit cDNA transfected in the cell could influence the amount of unassembled subunits remaining associated with calnexin; at the transfection ratios of β relative to α, δ, and γ used in this study, the β-subunit is not detected in association with calnexin in cells co-transfected with α, β, γ, and δ (Fig. 4B, lane 5). The detection of α- and δ-subunits associated with calnexin in cells co-transfected with α, γ, and δ (α-subunit, Fig. 4A, lane 4; δ-subunit, Fig. 4C, lane 4) and α, β, γ, and δ (α-subunit, Fig. 4A, lane 5; δ-subunit, Fig. 4C, lane 5) is most likely due to the presence of unassembled α- and δ-subunits associated with calnexin, since unassembled α-subunits have been detected in HEK cells co-transfected with α, γ, and δ and α, β, γ, and δ (Kreienkamp et al., 1995). Background signals caused by the nonspecific adsorption of receptor protein to the immunoprecipitation resin were not detected on Western blots (Fig. 4D, lane 3, for the α-subunit; similar results were obtained for the β- and δ-subunits, data not shown). Therefore all subunit protein bands displayed on these Western blots appear due to a specific antigen-antibody reaction.

Calnexin Is Not Detected in Association with αδ Dimers—To determine whether calnexin is associated with αδ dimers, plates of cells were transfected with α alone or co-transfected with α and δ, the expression levels of unassembled α-subunits and assembled αδ dimers were determined using 125I-Bgtx binding assays and detergent extracts derived from the same pool of cells were subjected to the calnexin co-immunoprecipitation procedure. A correlation in the expression levels estimated by 125I-Bgtx binding assays to the banding density of α-subunits co-immunoprecipitated with calnexin was used to determine whether calnexin associates with αδ dimers. The methods used to calculate expression of unassembled α-subunits and assembled αδ dimers are indicated in the legend to Fig. 5 and under “Experimental Procedures.” If calnexin were hypothetically associated with αδ dimers at an affinity equivalent to its association with α-subunit monomers, the α-subunit banding intensity for the αδ sample (Fig. 5B, lane 2) would be 4-fold denser than the sample expressing the α-subunit alone (Fig. 5B, lane 1), since the expression level of αδ dimers (Fig. 5A, bar 3) was empirically determined to be approximately 4-fold higher than the α-subunit expressed alone (Fig. 5A, bar 1). On the other hand, if calnexin were associated only with unassembled α-subunit monomers and not αδ dimers, the α-subunit band in the Western blot (Fig. 5B) should be denser in the α-subunit transfected alone configuration (Fig. 5B, lane 1) since its expression is 3-fold higher than unassembled α-subunits expressed in cells co-transfected with α and δ (Fig. 5A, bar 2). The appearance of a faint α-subunit band in the Western blot of the αδ co-transfected sample (Fig. 5B, lane 2) relative to the lane displaying the expression of the α-subunit alone (Fig. 5B, lane 1) indicates that calnexin association with αδ dimers is reduced appreciably or virtually non-existent.

Calnexin Association with α-Subunits Recognized by mAb 35—Nascent α-subunit polypeptide chains proceed through a folding and maturation pathway involving disulfide bond formation and glycosylation (Merlie and Smith, 1986; Miles and Huganir, 1988). The folded conformation of the α-subunit can form in the absence of neighboring receptor subunits (Blount and Merlie, 1988; Green and Claudio, 1993). The monoclonal antibody mAb 35 has been used extensively to monitor the folding of the α-subunit because it is believed to recognize only α-subunits folded into a native conformation (Tzartos et al., 1981). We performed co-immunoprecipitation experiments with mAb 35 and developed Western blots with calnexin antibody to determine whether calnexin associates with α-subunits recognized by mAb 35. Cells were transfected with α alone or co-transfected with α, β, γ, and δ. To verify that immunoprecipitation had occurred, the Western blot in Fig. 6A was first probed with mAb 210 which detects the α-subunit; α-subunit bands are present in the blot for the transfections of α alone (Fig. 6A, lane 3) and in the co-transfection of α, β, γ, and δ (Fig.
Calnexin is associated with α-subunits in their native conformation. Cells were transfected with α-subunit cDNA alone (15 µg of DNA/plate) or with cDNAs encoding α-, β-, and γ-subunits using 15 µg of DNA/plate α-subunit DNA and 7.5 µg of DNA/plate for the β-, γ-, and δ-subunits, and detergent extracts were immunoprecipitated (IP) with mAb 35. A, the Western blot was developed with mAb 210 which recognizes the α-subunit (EXT, detergent extract, RES, immunoprecipitation resin). Lane 1, mAb 35 alone, showing the location of the IgG heavy chain; lane 2, detergent extract of untransfected HEK cells not immunoprecipitated; lane 3, immunoprecipitated sample derived from cells transfected with the α-subunit alone; lane 4, immunoprecipitated sample derived from cells co-transfected with α, β, γ, and δ, B, the Western blot shown in panel A re-probed with antibody to calnexin (CLNX). Lane 5, untransfected HEK cell extract, without immunoprecipitation, exhibits a strong signal at approximately molecular mass 90 kDa, the approximate molecular mass of calnexin (Bergeron et al., 1994); lane 6, immunoprecipitated sample derived from cells transfected with α alone; lane 7, immunoprecipitated sample derived from cells transfected with α, β, γ, and δ, C, control for nonspecific adsorption of calnexin to the immunoprecipitation resin. The Western blot was developed with calnexin antibody. Lane 8, untransfected HEK cell extract without immunoprecipitation; lane 9, mock immunoprecipitation without antibody of the HEK cell extract. D, control for the cross-reactivity of mAb 35 with calnexin. Lane 10, untransfected HEK cell extract without immunoprecipitation; lane 11, mAb 35 immunoprecipitation of the detergent extract of untransfected HEK cells. This same blot was re-probed with the calnexin antibody and the presence of protein bands corresponding to the migration distance of calnexin in the transfection with α alone (Fig. 6B, lane 6) and the co-transfection with α, β, γ, and δ (Fig. 6B, lane 7) indicate that calnexin co-associates with native α-subunits. The association of calnexin with unassembled α-subunits in the co-transfection α, β, γ, and δ presumably contributes to the calnexin signal in Fig. 6B, lane 7, since it appears that unassembled α-subunits are associated with calnexin in the similar co-transfection shown in Fig. 4A, lane 5, where the antibody specificities for the α-subunit and calnexin in the immunoprecipitation and Western blot are reversed.

6A, lane 4). This same blot was re-probed with the calnexin antibody and the presence of protein bands corresponding to the migration distance of calnexin in the transfection with α alone (Fig. 6B, lane 6) and the co-transfection with α, β, γ, and δ (Fig. 6B, lane 7) indicate that calnexin co-associates with native α-subunits. The association of calnexin with unassembled α-subunits in the co-transfection α, β, γ, and δ presumably contributes to the calnexin signal in Fig. 6B, lane 7, since it appears that unassembled α-subunits are associated with calnexin in the similar co-transfection shown in Fig. 4A, lane 5, where the antibody specificities for the α-subunit and calnexin in the immunoprecipitation and Western blot are reversed.

The control for the nonspecific adsorption of calnexin to the immunoprecipitation resin indicates that calnexin does not adhere to the resin, since calnexin is absent in this sample (Fig. 6C, lane 9). There is no detectable cross-reactivity of mAb 35 with calnexin since mAb 35 does not immunoprecipitate calnexin (Fig. 6D, lane 11), indicating that the calnexin protein bands observed in the Western blots in Fig. 6B, lanes 6 and 7, are due to specific associations among mAb 35, α-subunits, and calnexin.

**DISCUSSION**

Using transiently expressed acetylcholine receptors in HEK-293 cells, we demonstrate here intracellular accumulation of fully assembled receptor complexes in brefeldin A-treated cells and the association of unassembled α-, β-, and δ-subunits with calnexin, a chaperone protein that transverses the ER membrane and possesses an ER retention motif (Bergeron et al., 1994). Our data indicate that these phenomena are involved in the biogenesis and expression of acetylcholine receptors transiently expressed in HEK-293 cells and complement other reports focusing on the biogenesis of the acetylcholine receptor, including those investigations which identified intermediates in the assembly pathway (Blount and Merlie, 1988; Green and Claudio, 1993; Kreienkamp et al., 1995), amino acid specificity at the contact interfaces between neighboring subunits (Verrall and Hall, 1992; Kreienkamp et al., 1995), and chaperone protein attachment to immaturely folded α-subunits (Blount and Merlie, 1991; Gelman et al., 1995). The processes controlling the biogenesis of the acetylcholine receptor, which has traditionally served as a research paradigm for other ligand-gated ion channels, are likely to have general applicability to the assembly of other ion channels and multisubunit transmembrane proteins.

Previous investigations indicated unassembled α-subunits are subject to relatively rapid degradation relative to more fully assembled intermediates (Merlie and Lindstrom, 1983; Blount and Merlie, 1990). We extended these observations to receptor subunits expressed in brefeldin A-treated cells and observed that assembly with the β-subunit enhances the intracellular expression levels of the δ-subunit as well as folded α-subunits. The elevated levels of receptor complex are most likely due to the resistance of the oligomers to protease degradation in the lumen of the ER, or the receptor subunits are no longer targeted to enter degradation pathways as seen with the rapid degradation of unassembled α-subunits (Blount and Merlie, 1990). Amino acid acid sequences that specify protein targeting into degradation pathways have been identified in the unassembled subunits of the T-cell receptor (Bonifacino et al., 1990; Bonifacino et al., 1991; Wileman et al., 1993), these sequences are believed to be covered when contacts are formed between neighboring subunits (Bonifacino et al., 1990). This phenomenon causes precursors of the assembled oligomers to have a higher propensity for degradation, an observation consistent with other studies on the acetylcholine receptor (Merlie and Lindstrom, 1983; Blount and Merlie, 1990).

The elevated expression of the α- and δ-subunits when co-expressed with the β-subunit is not accounted for by increased transcriptional or translational rates upon a two-subunit transfection because the number of 125I-α-Bgtx binding sites are similar when the α-subunit is expressed alone compared to the co-expression of the α- and β-subunits (Fig. 2C). The β-subunit does not appear to associate with the α-subunit in the absence of the δ- or γ-subunits in HEK cells, implicating assembly with the β-subunit as the factor that elevates the expression of the αβδ, and αβγδ configurations. At each assembly step there is an increased accumulation of more assembled intermediates, a process that should guide the assembly pathway in the direction of constructing the receptor pentamer.

Assembly of the α-, γ-, and δ-subunits with the β-subunit increases the intracellular pool of receptor protein available for export to the cell surface, an approximately 3-fold elevation of intracellular expression coincides with this (Fig. 2B). However, there is an approximately 10-fold elevation in expression at the cell surface upon addition of the β-subunit to the α-, γ-, and
δ-subunits (Fig. 1A), indicating that mechanisms beyond resisting intracellular degradation may also control expression at the cell surface. Another mechanism which may control expression at the cell surface is the presence of ER retention signals which sequester receptor precursors to the endoplasmic reticulum (Cosson and Letourneur, 1994; Letourneur et al., 1995), these sequestering signals are presumably removed or masked when the β-subunit associates with the receptor.

Calnexin has an ER retention motif in its cytoplasmic domain and its adhesion to the α-, β-, and δ-subunits may provide one level of retention of single subunits in the ER, in a manner similar to that demonstrated for the association of calnexin to unassembled T-cell receptor subunits (Rajagopalan et al., 1994). Overexpression of truncated calnexin that lacked the ER retention motif resulted in the export of these unassembled T-cell receptor subunits to the cell surface (Rajagopalan et al., 1994). Additional means for retention in the ER may also exist for acetylcholine receptor subunits because αδ dimers, whose association with calnexin was not detected, are retained in the ER. Assembly with the β-subunit may displace other chaperone proteins with ER retention signals associated with αδ dimers or mask ER retention motifs that are intrinsic to the amino acid sequences of the α- and/or δ-subunits.

Our observations indicating the pool of unassembled receptor subunits associated with calnexin is utilized as assembly proceeds from monomers to dimers implicates calnexin in the assembly process. The reductions in the subunit band density in Western blots, when co-immunoprecipitated receptor monomers are compared to receptor dimers (Fig. 5B) and other multiple subunit combinations (Fig. 4), indicate that calnexin-receptor subunit complexes are precursors of the receptor oligomer. Calnexin molecules are presumably displaced as two receptor subunits associate, or assembly promotes subunit folding resulting in the dissociation of calnexin.

On sucrose gradients with EDTA in the buffer, which precludes the adherence of calnexin (Bergeron et al., 1994), the slowest migrating peak on the density gradient yields an S value expected for a monomer species (Kreienkamp et al., 1995). When the receptor is modeled as a cylindrical structure with the circular orientation of subunits (Kreienkamp et al., 1995; Machold et al., 1995), dimer formation between α and δ involves the positive face of α and the negative face of δ. Assuming that calnexin recognizes homologous regions in the subunits, calnexin dissociation from the dimer may indicate that exposure of both the counterclockwise and clockwise faces of the receptor subunits are necessary for calnexin association. Oligosaccharide chains also appear to be involved in recognition by calnexin (Bergeron et al., 1994).

Alternative interpretations for the role of the transient association of calnexin with the receptor subunits include calnexin being primarily associated with misfolded receptor monomers which are not incorporated into mature receptors. If this interpretation were hypothetically correct, we would have observed in our co-immunoprecipitation and Western blot experiments similar receptor subunit banding intensities in samples derived from cells expressing one subunit to those co-expressing multiple receptor subunits, since α-subunit folding is believed to be independent of α-δ assembly (Blount and Merlie, 1991). In addition, calnexin is detected in association with α-subunits recognized by mAb 35, which recognizes maturely folded α-subunits (Tzartos et al., 1981), indicating that at least a portion of the α-subunit pool associated with calnexin is properly folded.

A previous study using embryonic chicken muscle cells (Gelman et al., 1995) indicated that calnexin was not appreciably associated with α-subunits recognized by mAb 35. The difference in findings between the two studies might be accounted for by the different conditions that influence sensitivity of detection, such as higher detergent concentrations for extraction which may have destabilized the calnexin-receptor subunit complex, double immunoprecipitation and elution steps which may have caused significant loss of protein prior to loading samples on gels, and the metabolic labeling of cells with [35S]methionine to detect protein bands as opposed to the use of a second antibody and the chemiluminescent protocol to detect proteins in the Western blots of our study. Observations derived from our expression system of mouse acetylcholine receptors in HEK cells appear most consistent with calnexin being primarily involved in stabilizing the receptor monomers for subunit assembly.

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