Characterization of Human Recombinant Annexin II Tetramer Purified from Bacteria: Role of N-Terminal Acetylation†

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ABSTRACT: Annexin II tetramer (AIIt) is a Ca^{2+}-dependent, phosphatidylserine-binding, and F-actin-bundling phosphoprotein which is localized to both the extracellular and cytoplasmic surfaces of the plasma membrane. The tetramer is composed of two p36 heavy chains and two p11 light chains. We have produced prokaryotic cDNA expression constructs for both p36 and p11. Both proteins were expressed in large amounts in *Escherichia coli* upon induction with IPTG. Electrospray ionization mass spectrometry and amino acid sequence analysis of purified recombinant p36 (rp36) and recombinant p11 (rp11) suggested that the recombinant proteins were identical to their native counterparts except for the lack of N-terminal acetylation of rp36. Furthermore, the non-acetylated rp36 bound rp11 and formed AIIt. The circular dichroism spectra and urea denaturation profiles of acetylated AIIt and non-acetylated rAIIt were identical. In addition, both the acetylated AIIt and non-acetylated rAIIt were similar in their Ca^{2+} dependence and concentration dependence of phospholipid liposome aggregation, chromaffin granule aggregation, and F-actin bundling. These results suggest that N-terminal acetylation of p36 is not in fact necessary for binding of the protein to p11 and that N-terminal acetylation does not affect the conformational stability of AIIt or the *in vitro* activities of AIIt. The availability of large amounts of rAIIt will facilitate further characterization of the structure−function relationships of the protein.

The annexins are a family of about thirteen proteins that bind to acidic phospholipids in a Ca^{2+}-dependent manner (see Swairjo & Seaton, 1994; Raynal & Pollard, 1994; Kaetzel & Dedman, 1995, for reviews). The amino acid sequence of the annexins indicates four repeats (eight repeats in the case of annexin VI) of about seventy amino acids which are highly homologous. Analysis of the crystal structure of several of the annexins has allowed conclusions to be made as to the structure of these proteins (Swairjo et al., 1995; Luecke et al., 1995; Favier-Perron et al., 1996). Each repeat comprises one compact domain which consists of five α-helices wound into a right-handed super-helix. The domains are arranged in a planar, cyclic array with a convex and a concave side. The convex side faces the biological membrane and contains the Ca^{2+} and phospholipid-binding sites. The concave side faces the cytosol and contains the N- and C-termini. The domains also appear to surround a hydrophilic pore which is an ion conductance channel (Demange et al., 1994). Although the *in vivo* function of the annexins is at present unclear, these proteins have been implicated in an array of physiological processes such as cell differentiation, mitogenesis, exocytosis, and endocytosis.

Annexin II (p36)[1] is unique among the annexins in that the N-terminus of the protein contains a high affinity binding site for a dimeric protein (monomeric M_r 11 000) which is a member of the S100 family of Ca^{2+}-binding proteins (reviewed in Waisman, 1995). The heterotetrameric complex formed by these proteins ($p36_2p11_2$) is referred to as annexin II tetramer (AIIt) and is the predominant form in most cells. AIIt was initially shown to be present at the cytosolic surface of the plasma membrane of many cells (Nigg et al., 1983; Greenberg & Edelman, 1983; Courtneidge et al., 1983; Glenney et al., 1987; Osborn et al., 1988; Nakata et al., 1990; Senda et al., 1994), and in the case of secretory cells such as the cells of the adrenal medulla or anterior pituitary (Nakata et al., 1990; Senda et al., 1994), AIIt has been shown to form cross-links between secretory granules and plasma membrane. This has led to the suggestion that AIIt is involved in exocytosis or endocytosis (reviewed in Waisman, 1995). Interestingly, in endothelial cells and several other cell lines, AIIt has been shown to exist on the extracellular surface of the plasma membrane (Chung & Erickson, 1994; Yeatman et al., 1993; Wright et al., 1994; Hajjar et al., 1994).

The two major mechanisms involved in the regulation of AIIt are the serine or tyrosine phosphorylation of the protein

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; AIIt, annexin II tetramer; rAIIt, annexin II tetramer formed from recombinant p36 and p11; p11, p11 light chain of annexin II tetramer; p36, p36 heavy chain of annexin II tetramer; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; Ac, acetyl; buffer A, 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 150 mM NaCl, and 0.1 mM EGTA; buffer B, 10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 0.5 mM EGTA; buffer C, 10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM $CaCl_2$; buffer D, 340 mosm/kg sucrose, 25 mM Hepes, pH 7.5; $\Delta\epsilon$, circular dichroic absorption coefficient ($\epsilon_L - \epsilon_R$); IPTG, isopropyl thio-β-D-galactoside; MES, 2-(*N*-morpholino)-ethanesulfonic acid; DIFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride.

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and the binding of the p11 subunit. AIIt is an *in vivo* substrate of protein kinase C and pp60^{src}, and the phosphorylation of AIIt has been shown to be a negative modulator of the *in vitro* activities of the protein (Johnstone et al., 1992; Hubaishy et al., 1995). Conversely, the binding of p11 subunit appears to direct the protein to the plasma membrane (Thiel et al., 1992).

Previous attempts at producing rAIIt from rp36 and rp11 subunits have been unsuccessful (Thiel et al., 1991). In the current report, we describe a recombinant bacterial expression system that allows the expression and purification of rp36 and rp11 on the order of milligrams. The recombinant proteins were similar in structure to their native counterparts except for the lack of N-terminal acetylation of the rp36 subunit. The formation of rAIIt by the recombinant subunits has allowed us to assess the role of N-terminal acetylation of p36 in the conformational stability and *in vitro* activities of AIIt. We found that N-terminal acetylation of p36 was not required for the conformational stability or Ca^{2+}- and concentration-dependent interaction of AIIt with phospholipid liposomes, secretory granules, or F-actin.

EXPERIMENTAL PROCEDURES

Materials. G-Actin was prepared from fresh rabbit skeletal muscle and purified by gel filtration on a Sephadex G-100 column (2.6 × 60 cm, Pharmacia) as previously described in Ikebuchi and Waisman (1990). G-Actin was converted to F-actin by addition of KCl and $MgCl_2$ to final concentrations of 50 and 1 mM, respectively, and stored at 1 mg/mL (4 °C) prior to use in F-actin bundling assays. Annexin II tetramer and annexin II monomer were prepared from bovine lung according to Khanna et al. (1990) and stored at −70 °C in 40 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 0.1 mM EGTA, and 150 mM NaCl. Affinity purified polyclonal antibodies to bovine p36 and bovine p11 were obtained from Biodesign International (Kennebunk, ME). Chromaffin granules were purified according to Jones et al. (1994), and the purified granules were washed twice (10000*g* for 40 min) in 100 mL of buffer D (340 mosm/kg sucrose, 25 mM Hepes, pH 7.5) and stored at between 4 and 8 mg/mL, in buffer D, at 4 °C. Phospholipid liposomes were prepared fresh daily according to Johnstone et al. (1993). Phosphatidylserine (PS) (400 mg) and diacylglycerol (DG) (40 mg) in 200 mL of chloroform were diluted with 4 volumes of methanol, dried under a stream of nitrogen, and sonicated (2 × 5 s bursts at 70 W with a Braun probe sonicator) in 1 mL of 25 mM Tris-HCl, pH 7.5. p36 human cDNA prokaryotic expression construct (pDS10-p36) containing the novel N-terminus, MRGSFKMS, was a generous gift from Dr. Volker Gerke (Max Planck Institute, Goettingen).

Expression of Annexin II Heavy Chain and p11 Light Chain in Escherichia coli. The cDNA sequences of human p11 and p36 subunits were pulled out of p11 human cDNA yeast expression construct YEp51-p11 (kindly donated by Dr. Carl Creutz, University of Virginia; Creutz et al., 1992) and p36 human cDNA expression construct pcDX-p36 (kindly donated by Dr. Tony Hunter, Salk Institute, personal communication) by PCR, respectively. PCR was performed in a Perkin-Elmer System 480 DNA Thermal Cycler using the following primers: 5′-ATGCCATCTCAAATGGAACACGCCATG-3′ (5′ primer) and 5′-CTACTTCTTTCCCTTCTGCTTCATGTGTACTACAAAATAG-3′ (3′ primer) for p11 and 5′-ATGTCTACTGTTCACGAAATCC-3′ (5′ primer) and 5′-TCAGTCATCTCCACCACACAG-3′ (3′ primer) for p36. These sequences are derived from the nucleotide sequence of human p11 and p36 reported by Kube et al. (1991) and Huang et al. (1986), respectively. The 50 μL reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KC1, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of each primer, 2.5 units of AmpTaq polymerase (Perkin-Elmer), and 5 ng of cDNA templates (YEp51-p11 and pcDX-p36). Denaturation of the sample was carried out at 94 °C for 2 min followed by 35 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C. The PCR was completed by having 1 cycle of 7 min of extension at 72 °C. A single PCR product of the expected size for p11 (294 base pairs) and p36 (1020 base pairs) was produced, as analyzed by agarose gel electrophoresis. The PCR product was blunt-end ligated to *Nde*I (Pharmacia) digested and Klenow fragment (Life Technologies, Inc.) filled-in pAED4.91 (kindly donated by Dr. Donald Doering, Massachusetts Institute of Technology) using a DNA ligation kit (Takara). The cDNAs of both pAED4.91-p11 and pAED4.91-p36 expression constructs were confirmed by DNA sequencing using the Perkin-Elmer/Applied Biosystems, Inc. (Mississauga, Ontario, Canada), automated fluorescent sequencing methodology.

Purification of Recombinant Human Annexin II Heavy Chain (p36) and p11 Light Chain. Culture (10 mL) of BL21 (DE3) *E. coli*, carrying the expression constructs (pAED4.91-p36 or pAED4.91-p11), was grown overnight at 37 °C in LB media containing 50 μg/mL ampicillin. The culture was added to 1 L of LB media with 50 μg/mL ampicillin and grown until the $A_{600\,nm}$ reached 0.8, after which the culture was induced for expression by addition of 0.8 mM IPTG. After 4 h of induction, the cells were harvested by centrifugation at 4000*g* for 30 min. The pellets were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 mM DIFP, and 10 μg/L leupeptin) using a French press (1000 psi). After centrifugation at 100000*g* the supernatant was dialyzed overnight against a dialysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT). The dialyzed rp36 sample was adjusted to pH 6.0 with solid MES and applied to a Fast-S column previously equilibrated with 50 mM MES, pH 6.0, and 1 mM DTT and eluted with a linear salt gradient (0−0.5 M NaCl), which resulted in the elution of rp36 at 350 mM NaCl. Fractions containing rp36 were dialyzed against dialysis buffer and applied to an affinity column of heparin−Sepharose, and the rp36 was eluted at 150 mM NaCl. The rp36 was then concentrated and applied to a Superose 12 column equilibrated with buffer A (40 mM Tris-HCl, pH 7.5, 1 mM DTT, 150 mM NaCl, and 0.1 mM EGTA). The rp36 eluted at a position on the Superose 12 column corresponding to a molecular weight of about 38 000 for a globular protein (Figure 2). The purified rp36 was collected and stored at −70 °C.

The initial steps for the purification of human rp11 were identical to the procedure used for purification of human rp36. The bacterial supernatant was centrifuged at 100000*g*, and the supernatant was dialyzed overnight against dialysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT). The supernatant was then applied to a Fast Q column equilibrated with the same dialysis buffer, and the unbound fraction was adjusted to pH 6.0 with solid MES and applied to a Fast-S

column previously equilibrated with 50 mM MES, pH 6.0 and 1 mM DTT. The rp11 was eluted with a linear salt gradient (0−0.5 M NaCl), which resulted in the elution of rp11 at 250 mM NaCl. Fractions containing rp11 were concentrated and applied to a Superose 12 column equilibrated with buffer A, and the pure rp11 was collected and stored at −70 °C. The molecular mass of the rp11 was determined by gel permeation chromatography to be about 27 000 for a globular protein (Figure 2). This was consistent with the rp11 forming a dimer as has been reported for the native p11 (Gerke & Weber, 1985).

Annexin II Tetramer Activity Assays. F-Actin bundling was assessed by measurement of the light scattering intensity perpendicular to the incident light in a Perkin-Elmer 650-10S spectrofluorimeter as detailed previously (Jones et al., 1992). The excitation and emission wavelengths were 400 nm with slit widths of 2 nm, and the light scattering of water was set to zero. F-Actin (1.16 μM) was incubated in bundling buffer (50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT, 0.5 mM $CaCl_2$, and 25 mM MES, pH 6.5) in the presence or absence of AIIt at 20 °C for 10 min in a final volume of 0.1 mL. Light scattering intensity was measured in a 3 × 3 × 25 mm microcuvette (Helma). Chromaffin granule aggregation was measured as described previously (Jones et al., 1994). The reaction mixture contained, in a final volume of 0.6 mL, purified chromaffin granules at an $A_{540\,nm}$ of 0.30, in 25 mM Hepes, pH 7.5, 20 μM $CaCl_2$, and sucrose at a final osmolality of 340 mosm/kg. Optical density of the aliquot was read prior to protein addition, and a final absorbance reading was taken after 15 min. Measurements were taken at a wavelength of 540 nm and at a temperature of 20 °C. Unless otherwise stated, addition of 0.56 μM AIIt initiated the reaction. Phospholipid liposomes were prepared as described by Nakata et al. (1990). Essentially, 50 μL of 20 mg/mL phosphatidylserine, phosphatidylethanolamine, and cholesterol in chloroform were dried in a N_2 stream and hydrated with 1 mL of a 30 mM Hepes, pH 7.5, and 2 mM $MgCl_2$. The mixture was sonicated (4 × 15 s bursts at 75 W with a Braun probe sonicator), and the phospholipid liposomes were stored at room temperature prior to their use in aggregation assays. Liposomal aggregation was measured at 20 °C and at a wavelength of 450 nm, in a final volume of 0.6 mL. The reaction mixture contained 30 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM Ca^{2+}, and sufficient liposomes such that the initial $A_{450\,nm}$ was 0.3. The $A_{450\,nm}$ of the reaction mixture was determined prior to protein addition, and a final reading was taken at 15 min.

Measurement of Protein Secondary Structure by Circular Dichroism. Circular dichroism (CD) measurements were performed with a Jasco J-715 spectropolarometer. Proteins (2.2 μM) were prepared in buffer B (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM DTT, and 0.5 mM EGTA). Samples (0.1 mL) were scanned in a quartz cuvette (0.5 mm path length) from 178 to 260 nm, using a bandwidth of 1 nm, a time constant of 4 s, and a scan speed of 10 nm/s. CD spectra of proteins were obtained by averaging four wavelength scans and were corrected by subtracting buffer scans. Results are expressed as $\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$).

Miscellaneous Techniques. Protein concentration was measured with the Bradford Coomassie Blue dye binding method using bovine serum albumin as a standard (Bradford, 1976). Alternatively, protein concentrations were determined spectrophotometrically using the following extinction coefficients (Hubaishy et al., 1995): G-actin, $A_{290\,nm} = 0.65$ for 1 mg/mL; AIIt, $A_{280\,nm} = 0.68$ for 1 mg/mL; p11, $A_{280\,nm} = 0.26$ for 1 mg/mL; annexin II, $A_{280\,nm} = 0.54$ for 1 mg/mL. Ca^{2+}-EGTA buffers were prepared according to Jones et al. (1994). Oligonucleotide primers were synthesized at the University Core DNA Services, The University of Calgary. ESI-MS was performed by Dr. Gilles Lajoie at the University of Waterloo Mass Spectroscopy Facility, Waterloo, Ontario. Molecular weights of recombinant proteins, deduced from the published cDNA sequences, were obtained from the SWISS-PROT database.

RESULTS

Expression and Purification of Human Recombinant p36 and p11. Previous expression of rp36 had utilized a strategy of inserting a *Bam*H1−*Hin*dIII cDNA fragment containing the entire coding sequence of p36 into the prokaryotic expression vector pDS10 (Thiel et al., 1991). Transformation of *E. coli* (JM 101) with this expression construct resulted in the expression of a recombinant p36 protein that contained six additional amino acid residues (MRGSFK) and the cDNA initiation codon-derived Met at the N-terminus. This novel N-terminus, MRGSFKMSTV, lacked the N-terminal acetyl-Ser which is present in the native protein and also introduced two basic residues (Arg and Lys) to the N-terminus. With this extended N-terminus, the recombinant p36 could not bind the p11 subunit and therefore did not form AIIt (Jost et al., 1994). It was unclear from this work if the absence of the N-terminal acetylation or the extension of the N-terminus by six foreign amino acids contributed to the inability of this recombinant p36 to bind p11.

Our approach was to blunt-end ligate p36 and p11 cDNA to *Nde*I digested and Klenow fragment filled-in pAED4.91. The rp36 expressed in *E. coli* by our procedure was soluble, which contrasted with a previous procedure that required the use of 8 M urea for extraction of the recombinant p36 from inclusion bodies (Thiel et al., 1991). As outlined under the Experimental Procedures, final purification of rp36 from bacterial lysates involved ion-exchange chromatography on Fast-S, affinity chromatography on heparin−Sepharose, and gel permeation chromatography on Superose 12. Typically, we obtained about 27 mg of pure rp36 from 1 L of bacterial culture. The rp36 was recognized by an affinity purified polyclonal antibody to bovine p36, and amino acid sequencing of the rp36 identified the N-terminus of the protein as STVHEIL. The M_r of human rp36 was determined to be 38 480.84 ± 7.24 by ESI-MS (Figure 1A), which was similar to the M_r 38 472 deduced from the cDNA sequence of initiating Met deleted, non-acetylated, human p36 (Huang et al., 1986). By comparison, the M_r of native bovine lung p36 was determined by ESI-MS to be 38 519.82 ± 3.29 (data not shown), which compares with the M_r 38 524 deduced from the cDNA sequence of initiating Met deleted, N-terminal acetylated, bovine p36 (Kristensen et al., 1986). These data therefore establish that the initiating Met of the rp36 was removed during expression of the protein and that, in contrast to the N-terminal Ser of native p36, the N-terminal Ser of rp36 is not acetylated.

Final purification of rp11 involved ion-exchange chromatography on Fast-Q and Fast-S and gel permeation chromatography on Superose 12 (see Experimental Procedures).

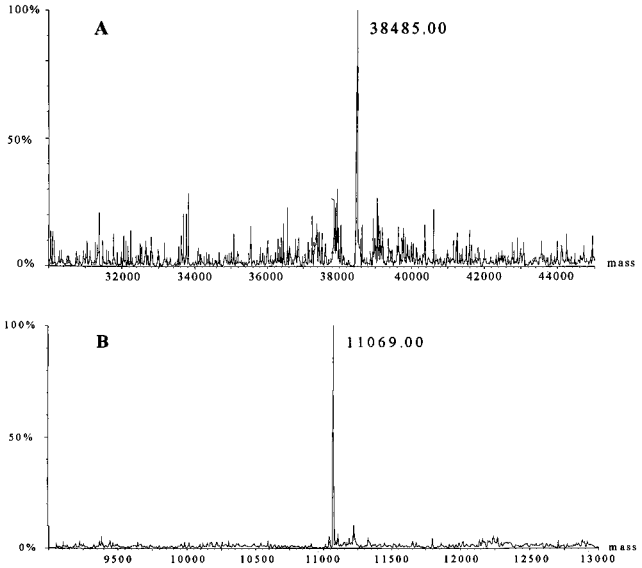

FIGURE 1: Determination of the molecular mass of rp36 and rp11 by ESI-MS. Real mass scale of the data for human rp36 (A) and human rp11 (B).

About 30 mg of pure p11 was purified from 1 L of bacterial culture. Gel permeation chromatography of rp11 on Superose 12 columns established that the recombinant protein existed as a dimer (see Experimental Procedures). Amino acid sequencing of the purified rp11 confirmed that the N-terminal sequence (PSQME...) of the recombinant protein is identical to that of the native p11. The M_r of rp11 was determined by ESI-MS as 11 068.28 ± 0.23 by ESI-MS (Figure 1B). This value was very similar to the M_r 11 069.38 ± 1.59 that we determined for the native p11 (data not shown) and to the M_r 11 072 deduced from the cDNA sequence of human p11 (Glenney & Tack, 1985). Our data therefore indicate that the initiating Met of rp11 has also been removed during the expression of the recombinant protein.

Analysis of the Secondary Structure of Native and Recombinant Annexin II. A potentially important feature of p36 is the N-terminal acetylation which has been suggested to be involved in the correct folding of the N-terminal region of p36 (Jost et al., 1994, 1992) and also to be involved in the binding of p11 (Becker et al., 1990). Both amino acid sequencing and mass spectrometry revealed that rp36 lacked N-terminal acetylation, the only respect in which it differed from native p36. We therefore investigated the role of N-terminal acetylation on the conformational stability and the Ca^{2+}-dependent binding of AIIt to phospholipid liposomes, secretory granules, and F-actin.

Since both native p36 and rp36 had identical Stokes radii as determined by chromatography on a calibrated Superose 12 column (data not shown), it was unlikely that the absence of N-terminal acetylation resulted in a gross conformational change in rp36. We also examined the relative conformational stability of native and rp36 by comparing the effect of a denaturing concentration of urea on the ellipticity at 222 nm. As shown in Table 1, the addition of 5 M urea to native p36 and rp36 resulted in a similar decrease in the ellipticity of the proteins of about 55−56%. This result strongly suggests that the N-terminal acetylation of p36 does not play a critical role in the conformational stability of the protein.

Table 1: Conformational Stability of Recombinant Proteins[a]

protein	decrease in $\Delta\epsilon_{222\ nm}$ (%)
native p36	55.1 ± 3.6
recombinant p36	55.9 ± 3.6
recombinant N-terminal extended p36	73.8 ± 2.7

[a] Recombinant proteins were incubated for 30 min at 20 °C in the presence or absence of 5 M urea in a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.5 mM EGTA. The results are expressed as the % decrease in $\Delta\epsilon_{222\ nm}$, where % decrease = $[\Delta\epsilon_{222\ nm}(0\ M\ urea) - \Delta\epsilon_{222\ nm}(5\ M\ urea)] \times 100/\Delta\epsilon_{222\ nm}(0\ M\ urea)$. Values are reported as mean ± SD ($n = 3$).

The original suggestion that N-terminal acetylation of p36 was required for the binding of p36 to p11 and might also contribute to the conformational stability of p36 N-terminus was based on the demonstration that a non-acetylated rp36 that contained a N-terminus that was extended by six amino acid residues (MRGSFK) from the initiating Met did not bind p11 (Jost et al., 1994, 1992; Thiel et al., 1991). We therefore tested the possibility that it was not the lack of N-terminal acetylation of the protein, but the addition of six amino acid residues to the N-terminus of the p36, that might be responsible for changes in the conformational stability of the protein. As shown in Table 1, the addition of 5 M urea to the N-terminal extended recombinant p36 caused a 74% decrease in the ellipticity of this protein. This would indicate that the N-terminal extended recombinant p36 was significantly less stable than either the native N-terminal acetylated p36 or our non-acetylated rp36. It therefore appeared that the extension of the N-terminus of p36 by the six amino acid residues resulted in a structurally destabilized protein and this destabilization was responsible for the inability of the p36 to bind p11.

Formation of Recombinant Annexin II Tetramer. The p11-binding site of p36 is located in the first 14 N-terminal residues of p36 (Johnsson et al., 1986; Glenney et al., 1986; Johnsson et al., 1988). This region of p36 forms an amphiphatic α-helix with hydrophobic side chains forming the contact site for p11. The N-terminal acetyl group of p36 is believed to be important for p11 binding, and studies with peptides to the N-terminal region of p36 have established that in the absence of the *N*-acetyl group the K_d of binding is increased by 1000-fold (Johnsson et al., 1988). Since our rp36 is identical to its authentic counterpart except for the lack of N-terminal acetylation, we were able to investigate the role of N-terminal acetylation in the binding of p36 to p11. Therefore, rp36 and rp11 were concentrated by ultrafiltration to about 5−8 mg/mL, and the proteins were combined and applied to a calibrated Superose 12 gel permeation column. As shown in Figure 2, two protein peaks were resolved by gel permeation chromatography. The minor protein peak that was resolved by gel permeation chromatography of mixtures of rp36 and rp11 moved with a mobility consistent with a M_r of 38 000. This suggested that the minor protein peak contained rp36; however, SDS−PAGE of the minor protein peak established the presence of both proteins. Rechromatography of the pooled minor protein peak on the Superose 12 column resulted in a single protein peak of relative mobility identical to that of the original minor protein peak (data not shown): i.e., the rp36 and rp11 in the minor protein peak did not form rAIIt. This indicated that a small portion of purified rp36 and rp11 could not combine to form rAIIt. The most plausible explanation

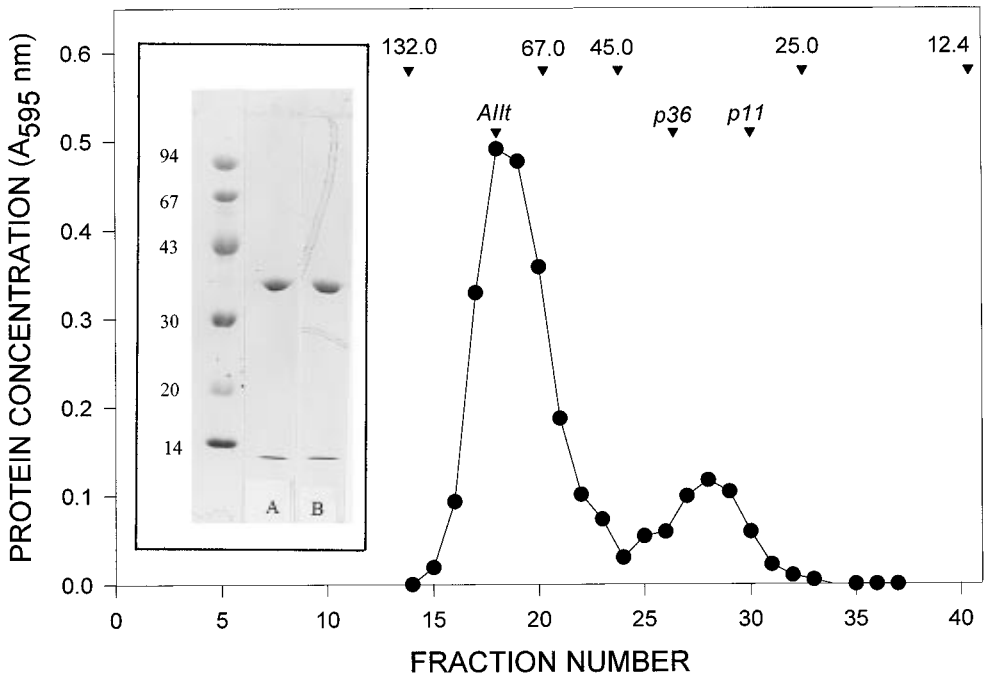

FIGURE 2: Non-acetylated rp36 binds rp11 and forms a tetramer. rp36 (0.260 μmol) and rp11 (0.258 μmol) were incubated in 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.15 M NaCl, and 0.1 mM EGTA for 10 min at 4 °C. The mixture was applied to a Superose 12 gel permeation column equilibrated with the same buffer, and fractions were analyzed for protein concentration. The position in the elution profile of several globular proteins of known molecular weight is provided. For comparison, the position in the elution profile of bovine lung AIIt (*AIIt*), rp36 (*p36*), and rp11 (*p11*) is also indicated. Inset: SDS−PAGE analysis of pooled rAIIt (fractions 18−21) (A) and bovine lung AIIt (B).

is that a small amount of these proteins were denatured either during purification or during the concentration of the proteins prior to their combination and application to gel permeation chromatography columns.

The major protein peak moved with a relative mobility identical to that of native AIIt, and the SDS−PAGE analysis confirmed that this protein peak contained both rp36 and rp11 (Figure 2, inset). Rechromatography of the pooled rAIIt on a Superose 12 column resulted in the appearance of a single protein peak, suggesting that the rAIIt did not dissociate into its subunits under these conditions. Our results therefore establish that non-acetylated p36 can bind p11 and form a tetramer.

Comparison of the Secondary Structures of Native and Recombinant AIIt. Since our rp36 and rp11 form a tetramer, it was important to determine if the secondary structure of the rAIIt was affected by the lack of N-terminal acetylation of the p36 subunit. CD is a sensitive technique for conformational analysis of proteins because the CD bands are characteristic of various secondary structures. Figure 3 compares the CD profiles of native AIIt and rAIIt. The CD scans of these proteins are virtually identical. Both proteins show identical minima at 222 and 208 nm and a maximum at 197 nm (Table 2).

Urea denaturation curves are commonly used to obtain an estimate of the conformational stability of proteins. The $[urea]_{0.5}$ from urea denaturation curves indicates the net conformational stability of the protein, that is, the sum of the stabilizing effects of hydrophobic interactions and electrostatic attractions. Therefore, the $[urea]_{0.5}$ from urea denaturation of AIIt would be expected to measure not only the stabilizing effects of intermolecular interactions of the individual p36 and p11 subunits but also the stabilizing

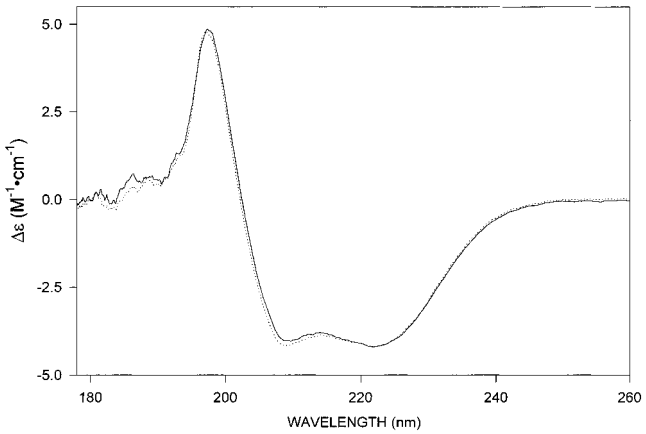

FIGURE 3: CD spectra of the native AIIt and rAIIt. Wavelength scans were conducted at 20 °C in a buffer C (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM Ca^{2+}). The protein concentration of native AIIt and rAIIt was 2.2 μM. (···) Native AIIt; (−) rAIIt.

Table 2: Comparison of CD Absorption Maximum and Minima of Native and Recombinant AIIt[a]

	native AIIt	recombinant AIIt
$\Delta\epsilon_{222\ nm}$	-4.18 ± 0.05	-4.20 ± 0.09
$\Delta\epsilon_{208\ nm}$	-4.11 ± 0.18	-3.94 ± 0.21
$\Delta\epsilon_{197\ nm}$	4.77 ± 0.63	4.74 ± 0.39

[a] CD scans were performed with 2.2 μM AIIt, at 20 °C and in a buffer containing 10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM $CaCl_2$.

effects of the p36−p11 interactions. As shown in Figure 4, the urea denaturation profiles of native AIIt and rAIIt are indistinguishable. The $[urea]_{0.5}$ values were calculated to be 5.35 ± 0.04 (mean ± SD, $n = 3$) for native AIIt and 5.38 ± 0.07 (mean ± SD, $n = 3$) for rAIIt.

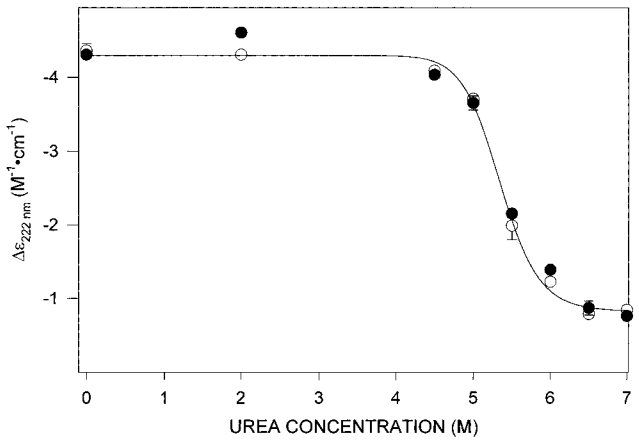

FIGURE 4: Urea denaturation of native AIIt and rAIIt. Native AIIt (○) and rAIIt (●) at final concentrations of 2.2 μM were prepared in buffer B and diluted with various volumes of 8 M urea dissolved in the same buffer to give the desired final concentration of urea. The samples were incubated at 20 °C for 60 min before their ellipticity at 222 nm was recorded. Results are expressed as mean ± SD ($n = 3$).

Collectively, the results obtained from comparisons of both the CD spectra and urea denaturation curves of native AIIt and rAIIt suggest that N-terminal acetylation of p36 does not exert a major influence on the structure of p36. The conformational stability and secondary structure of the non-acetylated protein are identical to those of the acetylated protein, and the non-acetylated rp36 binds to rp11 and forms the rAIIt.

Aggregation of Phospholipid Liposomes by Native and Recombinant AIIt. All annexins bind Ca^{2+} and phospholipids although individual annexins differ markedly in their affinities for these ligands (Blackwood & Ernst, 1990). Of the annexins tested to date, only annexin I and annexin II possess the ability to aggregate phospholipid liposomes at micromolar Ca^{2+} concentrations. The interaction of AIIt with Ca^{2+} and phospholipid liposomes has been shown to result in a large conformational change in the protein (Follenius-Wund et al., 1993). Furthermore, the binding of p11 to p36 results in a decrease of the K_d (Ca^{2+}) for phospholipid binding and aggregation (Powell & Glenney, 1987).

Figure 5 compares the concentration dependence of phospholipid aggregation by native AIIt and rAIIt. The relative potencies of both proteins for phospholipid aggregation are very similar, with values of $A_{0.5}$ (native AIIt) of 0.065 ± 0.003 μM (mean ± SD, $n = 3$) and $A_{0.5}$ (rAIIt) of 0.050 ± 0.005 μM (mean ± SD, $n = 3$). Native AIIt and rAIIt were also capable of aggregating the phospholipid liposomes to a similar extent, with A_{max} values of 228 ± 2% (mean ± SD, $n = 3$) and 236 ± 4% (mean ± SD, $n = 3$), respectively.

We also examined the Ca^{2+} dependence of phospholipid aggregation by recombinant and native proteins (Figure 6). rp36 was found to aggregate phospholipid liposomes with an $A_{0.5}$ (pCa^{2+}) of 6.08 ± 0.01. The binding of rp11 to rp36 increased the $A_{0.5}$ (pCa^{2+}) of the complex to 6.59 ± 0.07 (mean ± SD, $n = 3$). The $A_{0.5}$ (pCa^{2+}) of the rAIIt was also very similar to the $A_{0.5}$ (pCa^{2+}) of 6.71 ± 0.09 determined for the native protein. The steepness in the Ca^{2+} titration curves showed considerable experimental variation and did not allow conclusions to be made as to whether or nor these proteins differed in their apparent cooperativities with respect to Ca^{2+}.

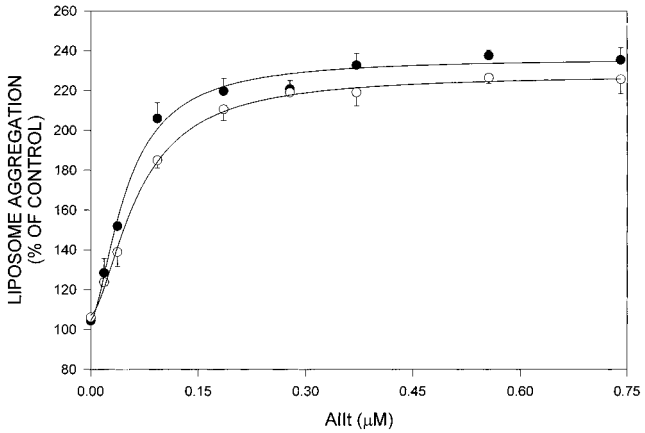

FIGURE 5: Concentration dependence of phospholipid liposome aggregation by native AIIt and rAIIt. Phospholipid liposomes consisting of phosphatidylserine, phosphatidylethanolamine, and cholesterol were prepared as per Experimental Procedures and then incubated at 20 °C with 30 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.2 mM $CaCl_2$. Immediately after taking the first reading of optical density, native AIIt (○) or rAIIt (●) was added to the reaction mixture at the concentrations indicated and the $A_{450\ nm}$ was determined after 15 min. Results are expressed as a percentage of starting $A_{450\ nm}$ (no added AIIt). Data shown are expressed as mean ± SD ($n = 3$).

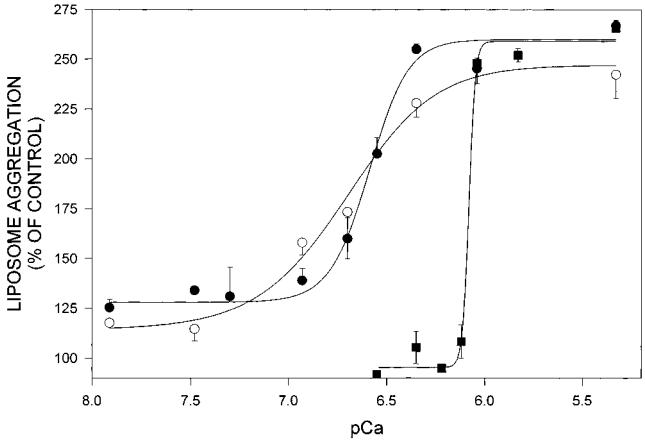

FIGURE 6: Ca^{2+} dependence of AIIt-dependent phospholipid liposome aggregation. Phospholipid liposomes consisting of phosphatidylserine, phosphatidylethanolamine, and cholesterol were prepared as per Experimental Procedures and then incubated at 20 °C with 30 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, and Ca^{2+} at the indicated concentrations. Immediately after taking the first reading of optical density, 0.56 μM native AIIt (○), 0.56 μM rAIIt (●), or 1.35 μM rp36 (■) was added to the reaction mixture at the concentrations indicated, and the $A_{450\ nm}$ was determined after 15 min. Results are expressed as a percentage of starting $A_{450\ nm}$ (no added AIIt). Data shown are expressed as mean ± SD ($n = 3$).

Collectively, these results indicate that the absence of N-terminal acetylation from the rp36 subunit or rAIIt does not affect the potency or Ca^{2+} requirement of rAIIt for phospholipid liposomal aggregation. Therefore, the recombinant protein appears to be very similar to the native protein with respect to its ability to interact with phospholipid liposomes.

Aggregation of Chromaffin Granules by Native and Recombinant AIIt. The interaction of annexins with model biological membranes such as secretory vesicle membranes (chromaffin granules) suggests a role for these proteins in membrane trafficking events such as exocytosis or endocytosis. The ability of AIIt to aggregate chromaffin granules at low micromolar Ca^{2+} concentration is a property unique

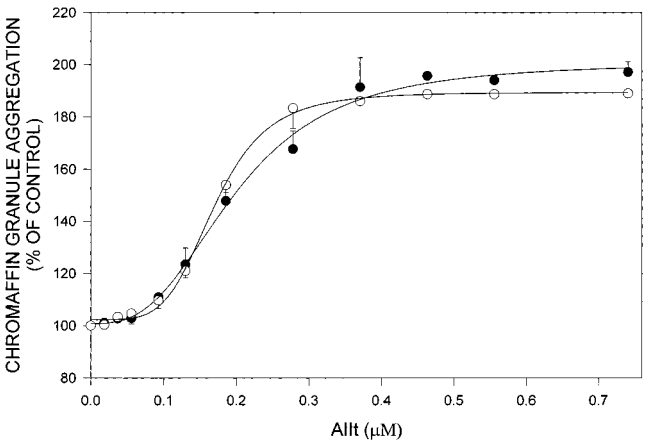

FIGURE 7: Concentration dependence of chromaffin granule aggregation by native AIIt and rAIIt. Chromaffin granules were purified as per Experimental Procedures and then incubated at 20 °C with 25 mM Hepes, pH 7.5, 20 μM $CaCl_2$, 50 mM KCl, and sucrose to a final concentration of 340 mosm. Immediately after taking the first reading of $A_{540\ nm}$, varying amounts of native AIIt (○) or rAIIt (●) were added to initiate the reaction, and the $A_{540\ nm}$ was determined after 15 min. Changes at $A_{540\ nm}$ are expressed as a percentage of starting $A_{540\ nm}$ (no added AIIt), where % increase = $[A_{540\ nm}(15\ min, +AIIt) - A_{540\ nm}(15\ min, -AIIt)] \times 100/A_{540\ nm}$ (0 min, −AIIt). Data are expressed as mean ± SD, $n = 3$.

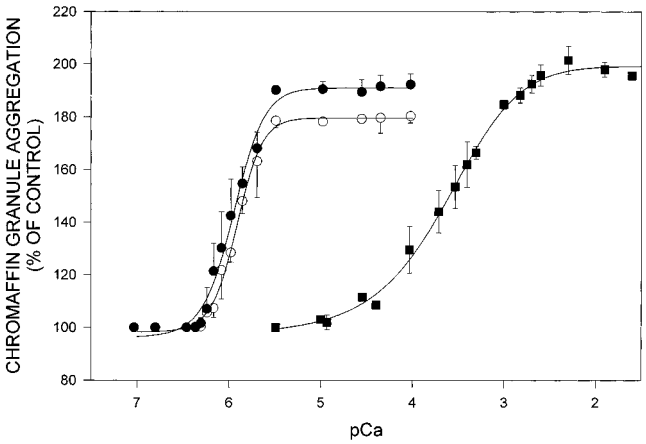

FIGURE 8: Ca^{2+} dependence of chromaffin granule aggregation by native AIIt and rAIIt. Chromaffin granules were purified as per Experimental Procedures and then incubated at 20 °C with 25 mM Hepes, pH 7.5, 50 mM KCl, sucrose to a final concentration of 340 mosm, and Ca^{2+} at the concentrations indicated. Immediately after taking the first reading of $A_{540\ nm}$, 0.56 μM native AIIt (○), 0.56 μM rAIIt (●), or 1.35 μM rp36 (■) was added to initiate the reaction, and the $A_{540\ nm}$ was determined after 15 min. Changes at $A_{540\ nm}$ are expressed as a percentage of starting $A_{540\ nm}$ (no added AIIt) as described in the legend to Figure 7.

among the annexins. Creutz's laboratory first demonstrated that p36, isolated by dissociation of purified bovine intestinal epithelial AIIt subunits with guanidine hydrochloride, was unable to aggregate chromaffin granules. This contrasted with the ability of AIIt to aggregate chromaffin granules at a pCa^{2+} of 5.74 (Drust & Creutz, 1988). Since the p11 subunit did not bind to or aggregate chromaffin granules, it was concluded that the binding of the p11 subunit induced a conformational change in the p36 subunit that resulted in a decrease in the Ca^{2+} requirement for chromaffin granule aggregation.

Figure 7 presents a comparison of the concentration dependence of chromaffin granule aggregation by native AIIt and rAIIt. The relative potencies of both proteins for chromaffin granule aggregation are very similar, with values of $A_{0.5}$ (native AIIt) of 0.185 ± 0.031 μM (mean ± SD, n = 3) and $A_{0.5}$ (rAIIt) of 0.192 ± 0.024 μM (mean ± SD, n = 3). Native AIIt and rAIIt were also capable of aggregating the chromaffin granules to a similar extent, with A_{max} values of 189 ± 1% (mean ± SD, $n = 3$) and 201 ± 3% (mean ± SD, $n = 3$), respectively. Although the rp36 subunit was able to aggregate chromaffin granules, this event required millimolar Ca^{2+} (Figure 8). Since the aggregation of chromaffin granules by rp36 subunit required millimolar Ca^{2+} and the chromaffin granule aggregation experiments were performed in the presence of 20 μM Ca^{2+}, one would expect that if dissociation of rAIIt had occurred during the binding to or aggregation of the chromaffin granules, the potency of the rAIIt would be appreciably less than that of the native AIIt. Therefore, under these experimental conditions, it was possible to conclude from the similar potencies of native AIIt and rAIIt that significant dissociation of the rAIIt into rp11 and rp36 subunits had not occurred.

Chromaffin granule aggregation is a sensitive assay for analysis of the functional integrity of the N-terminus of p36. Drust and Creutz (1988) have demonstrated that proteolysis of the N-terminus of p36 resulted in a dramatic change in the Ca^{2+} dependence of chromaffin granule aggregation. Furthermore, the binding of p11 to p36 was also shown to cause a large decrease in the Ca^{2+} requirement for chromaffin granules aggregation by p36. As shown in Figure 8, rp36 aggregates chromaffin granules with an $A_{0.5}$ (pCa^{2+}) of 3.63 ± 0.03 (mean ± SD, $n = 5$). The binding of rp11 to rp36 and formation of rAIIt resulted in an increase of the $A_{0.5}$ (pCa^{2+}) of chromaffin granule aggregation to 5.95 ± 0.02 (mean ± SD, $n = 5$). Furthermore, the $A_{0.5}$ (pCa^{2+}) of chromaffin granule aggregation by the native AIIt [5.91 ± 0.01 (mean ± SD, $n = 5$)] was very similar to the $A_{0.5}$ (pCa^{2+}) of rAIIt.

These results indicate that the N-terminal acetylation of the p36 subunit of AIIt does not influence the interaction of AIIt with chromaffin granules. Furthermore, the absence of N-terminal acetylation from the p36 subunit does not affect the p11-induced decrease in the Ca^{2+} requirement of p36-dependent chromaffin granule aggregation.

F-Actin Bundling Properties of Native and Recombinant AIIt. AIIt binds to F-actin in the presence of Ca^{2+} with a K_d (AIIt) of 0.23 μM and a stoichiometry of 1:1.9, AIIt:F-actin (Ikebuchi & Waisman, 1990). The binding of AIIt to F-actin results in the formation of large anisotropic bundles comprised of parallel arrays of closely packed filaments (Gerke & Weber, 1984; Ikebuchi & Waisman, 1990; Regnouf et al., 1991). The AIIt-dependent formation of anisotropic F-actin bundles is rapid and readily reversed by the addition of excess EGTA. The affinity and stoichiometry of the AIIt−F-actin interaction is similar to that of other F-actin bundling proteins, which suggests the physiological relevance of this property of AIIt. Although AIIt is similar to other known F-actin bundling proteins in terms of its binding characteristics, it is distinct from these bundling proteins in that it is the only F-actin bundling protein that is stimulated by increased Ca^{2+}.

Figure 9 presents the concentration dependence of F-actin bundling by native AIIt and rAIIt. Both proteins bundled F-actin to a similar maximal extent. Half-maximal F-actin bundling occurred at about 0.310 ± 0.020 μM native AIIt (mean ± SD, $n = 3$) or 0.291 ± 0.021 μM rAIIt (mean ±

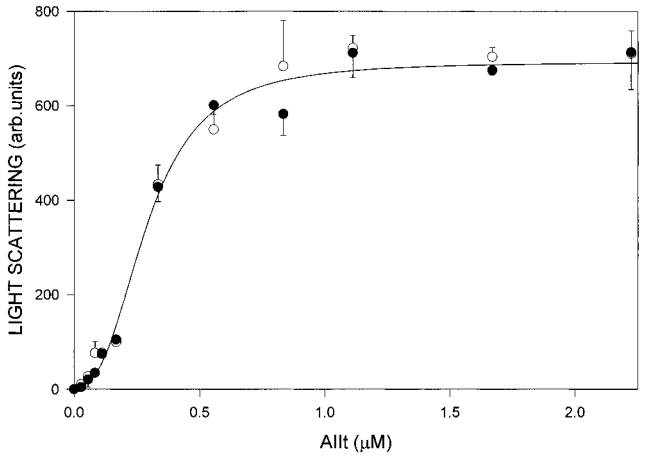

FIGURE 9: Concentration dependence of F-actin bundling by native AIIt and rAIIt. F-Actin (1.16 μM) was preincubated for 10 min at 20 °C in a buffer containing 25 mM MES, pH 6.5, 50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT, and 0.5 mM $CaCl_2$. Native AIIt (○) or rAIIt (●) at the concentrations shown was added to the reaction mixtures. After incubation at 20 °C for 10 min, aliquots of the reaction mixture were analyzed for F-actin bundling activity by light scattering.

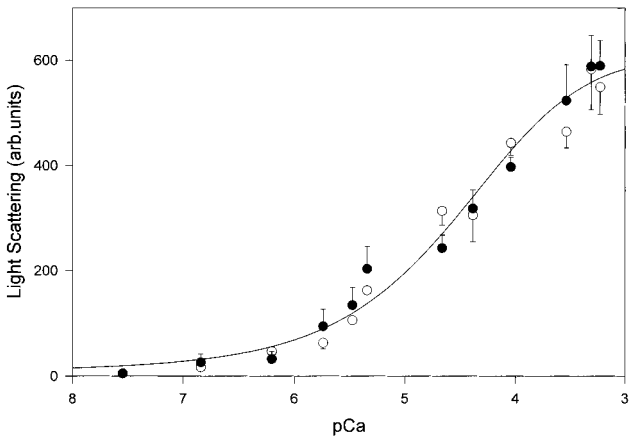

FIGURE 10: Ca^{2+} dependence of F-actin bundling by native AIIt and rAIIt. F-Actin (1.16 μM) was incubated for 10 min at 20 °C in a buffer containing 25 mM MES, pH 6.5, 50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT, and Ca^{2+} at the concentrations indicated. Native AIIt (○) and rAIIt (●), both at 1.4 μM, were added to initiate the reaction. After incubation, aliquots were analyzed for F-actin bundling activity by light scattering.

SD, $n = 3$), which indicated that native and recombinant proteins had similar F-actin bundling properties. We also examined the Ca^{2+} dependence of AIIt-dependent F-actin bundling. As shown in Figure 10, native AIIt and rAIIt had similar Ca^{2+} requirements for F-actin bundling. Native AIIt bundled F-actin with an $A_{0.5}$ (pCa^{2+}) of 4.580 ± 0.081 (mean ± SD, $n = 3$), while rAIIt bundled F-actin with an $A_{0.5}$ (pCa^{2+}) of 4.515 ± 0.076 (mean ± SD, $n = 3$). Consistent with our previous results, we were unable to detect significant bundling of F-actin by native p36 or rp36. Therefore, since the rp36 did not bundle F-actin (data not shown), while the rAIIt exhibited F-actin bundling properties very similar to the native AIIt, our results indicate that the binding of the p11 subunit to the p36 subunit conferred F-actin bundling activity to the p36 subunit.

DISCUSSION

The overall objective of these experiments was to elucidate the experimental conditions that would allow the production of a rAIIt that was structurally and functionally similar to the native protein. Although both p36 and p11 had been previously produced in the bacterial expression system (Thiel et al., 1991), the p36 subunit isolated by this approach was functionally compromised and could not bind to the p11 subunit. It was concluded that the lack of N-terminal acetylation of the p36 subunit was responsible for the inability of the p36 subunit to bind to the p11 subunit. However, the strategy used by this group for producing a cDNA expression construct resulted in the addition of six amino acid residues encoded by the expression vector to the N-terminus of the p36. However, the possibility that the introduction of these six amino acid residues to the N-terminus of the p36 might result in the disruption of the p36 conformational stability was not investigated.

We therefore began to investigate the possibility that bacterially expressed rp36 and rp11 subunits could be combined to form an active rAIIt. Our cloning strategy did not result in the addition of amino acid residue(s) to the N-terminus of the rp36 subunit. Since our rp36 and rp11 subunits were identical to the native proteins except for the lack of N-terminal acetylation of the rp36 subunits as confirmed by ESI-MS, we decided to examine whether or not N-terminal acetylation of p36 was critical for the binding of the protein to rp11. As shown in Figure 2, the non-acetylated rp36 binds to the rp11 and forms rAIIt. Therefore, the previous conclusion that N-terminal acetylation of p36 was necessary for p11 binding was incorrect. As shown in Table 1, our non-acetylated rp36 and acetylated native p36 appeared very similar in their conformational stability to urea denaturation, while non-acetylated N-terminal-extended recombinant p36 was less stable to urea denaturation. These results therefore indicated that the addition of the six amino acid residues to the N-terminus of p36 might be responsible for blocking p11 binding.

Our strategy for the expression of rp36 and rp11 involved the transformation of BL21 (DE3) *E. coli* with pAED4.91-p36 and pAED4.91-p11 expression constructs. This resulted in the expression of both rp36 and rp11 at high concentrations in *E. coli*.

We have examined four different aspects of AIIt structure and activity. First, the overall conformation of acetylated and non-acetylated AIIt was examined by comparison of the CD spectra of native AIIt and rAIIt. Since the CD spectra of both proteins were identical, we concluded that the lack of acetylation did not appear to dramatically affect the secondary structure of the proteins. Second, we examined the conformational stability of both proteins by comparing the urea denaturation profiles of the acetylated and non-acetylated proteins. Since the acetylated and non-acetylated proteins had similar $[urea]_{0.5}$ values, we concluded that acetylation did not appear to affect the conformational stability of AIIt. Third, several of the *in vitro* activities of acetylated and non-acetylated proteins were compared. We were unable to demonstrate any significant difference in the concentration dependence or Ca^{2+} dependence of AIIt-dependent phospholipid liposome aggregation, chromaffin granule aggregation, or F-actin bundling. Again, this indicated that acetylation did not influence the expression of several *in vitro* activities. Fourth, we examined the possible influence of N-terminal acetylation of p36 on the p11-induced conformational change in p36. As established earlier, the Ca^{2+} dependence of chromaffin granule aggrega-

tion by p36 and AIIt is dramatically different. Native p36 aggregates chromaffin granules with an $A_{0.5}$ (Ca^{2+}) of about 1 mM while native AIIt requires only about 2 μM Ca^{2+}. These results have been interpreted to suggest that the binding of p11 to p36 influences the interaction of p36 with several of its ligands by producing a conformational change in p36. Similarly, we found that the binding of rp11 to rp36 results in a decrease in the $A_{0.5}$ (Ca^{2+}) for chromaffin granule aggregation from 0.23 mM for rp36 to 1.0 μM for rAIIt. We also found that the binding of rp11 to rp36 results in a decrease in the $A_{0.5}$ (Ca^{2+}) of phospholipid liposome aggregation from 0.83 μM for rp36 to 0.26 μM for rAIIt. In the case of the F-actin bundling activity of AIIt, the role of p11 binding is most dramatic. Individually, p11 and p36 are incapable of significant bundling of F-actin; however, the binding of p11 to p36 activates the F-actin bundling activity of the p36 subunit. These results indicate that acetylation of the p36 subunit does not affect the p11-dependent regulation of several *in vitro* activities of p36.

The exact function of N-terminal acetylation of proteins is unclear and may vary depending on the protein involved. It has been suggested that N-terminal acetylation may play an important role in the biological function of many proteins. For example, the N-terminal acetylation of tropomyosin is required for the its binding to actin (Heald & Hitchcock-DeGregori, 1988; Urbancikova & Hitchcock-DeGregori, 1994). In contrast, many non-acetylated recombinant proteins are fully active. For example, the N-terminal acetylation of chaperonin 10 protein is not necessary for the correct folding of the protein and is also not important for chaperonin activity or mitochondrial import (Ryan et al., 1995). Similarly, other proteins which normally contain an acetylated N-terminus, such as human protein methyltransferase (MacLaren & Clarke, 1995), *N*-acetyltransferase type 1 (Ward et al., 1995), actin (Cook et al., 1991), liver transglutaminase (Ikura et al., 1990), thioltransferase (Yang & Wells, 1990), alcohol dehydrogenase (Hoog et al., 1987), and troponin C (Xu & Hitchcock-DeGregori, 1988), are stable and fully functional without an acetylated N-terminus. Our results indicate that AIIt belongs to the latter group of proteins for which N-terminal acetylation does not affect the *in vitro* activities or conformational stability of the protein.

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REFERENCES

Becker, T., Weber, K., & Johnsson, N. (1990) *EMBO J. 9*, 4207−4213.

Blackwood, R. A., & Ernst, J. D. (1990) *Biochem. J. 266*, 195−200.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Chung, C. Y., & Erickson, H. P. (1994) *J. Cell Biol. 126*, 539−548.

Cook, R. K., Sheff, D. R., & Rubenstein, P. A. (1991) *J. Biol. Chem. 266*, 16825−16833.

Courtneidge, S., Ralston, R., Alitalo, K., & Bishop, J. M. (1983) *Mol. Cell Biol. 3*, 340−350.

Demange, P., Voges, D., Benz, J., Liemann, S., Gottig, P., Berendes, R., Burger, A., & Huber, R. (1994) *Trends. Biochem. Sci. 19*, 272−276.

Drust, D. S., & Creutz, C. E. (1988) *Nature 331*, 88−91.

Favier-Perron, B., Lewit-Bentley, A., & Russo-Marie, F. (1996) *Biochemistry 35*, 1740−1744.

Follenius Wund, A., Pigault, C., & Gerard, D. (1993) *Biochem. Mol. Biol. Int. 29*, 653−660.

Gerke, V., & Weber, K. (1984) *EMBO J. 3*, 227−233.

Gerke, V., & Weber, K. (1985) *J. Biol. Chem. 260*, 1688−1695.

Glenney, J. R., Jr., & Tack, B. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7884−7888.

Glenney, J. R., Jr., Boudreau, M., Galyean, R., Hunter, T., & Tack, B. (1986) *J. Biol. Chem. 261*, 10485−10488.

Glenney, J. R., Jr., Tack, B., & Powell, M. A. (1987) *J. Cell Biol. 104*, 503−511.

Greenberg, M. E., & Edelman, G. M. (1983) *Cell 33*, 767−779.

Hajjar, K. A., Jacovina, A. T., & Chacko, J. (1994) *J. Biol. Chem. 269*, 21191−21197.

Heald, R. W., & Hitchcock-DeGregori, S. E. (1988) *J. Biol. Chem. 263*, 5254−5259.

Hoog, J. O., Weis, M., Zeppezauer, M., Jornvall, H., & von Bahr-Lindstrom, H. (1987) *Biosci. Rep. 7*, 969−974.

Huang, K. S., Wallner, B. P., Mattaliano, R. J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L. K., Chow, E. P., Browning, J. L., Ramachandran, K. L., Tang, J., Smart, J. E., & Pepinsky, R. B. (1986) *Cell 46*, 191−199.

Hubaishy, I., Jones, P. G., Bjorge, J., Bellagamba, C., Fitzpatrick, S., Fujita, D. J., & Waisman, D. M. (1995) *Biochemistry 34*, 14527−14534.

Ikebuchi, N. W., & Waisman, D. M. (1990) *J. Biol. Chem. 265*, 3392−3400.

Ikura, K., Tsuchiya, Y., Sasaki, R., & Chiba, H. (1990) *Eur. J. Biochem. 187*, 705−711.

Johnsson, N., Vandekerckhove, J., Van-Damme, J., & Weber, K. (1986) *FEBS Lett. 198*, 361−364.

Johnsson, N., Marriott, G., & Weber, K. (1988) *EMBO J. 7*, 2435−2442.

Johnstone, S. A., Hubaishy, I., & Waisman, D. M. (1992) *J. Biol. Chem. 267*, 25976−25981.

Johnstone, S. A., Hubaishy, I., & Waisman, D. M. (1993) *Biochem. J. 294*, 801−807.

Jones, P. G., Moore, G. J., & Waisman, D. M. (1992) *J. Biol. Chem. 267*, 13993−13997.

Jones, P. G., Fitzpatrick, S., & Waisman, D. M. (1994) *Biochemistry 33*, 13751−13760.

Jost, M., Thiel, C., Weber, K., & Gerke, V. (1992) *Eur. J. Biochem. 207*, 923−930.

Jost, M., Weber, K., & Gerke, V. (1994) *Biochem. J. 298 Pt 3*, 553−559.

Kaetzel, M. A., & Dedman, J. R. (1995) *News Physiol. Sci. 10*, 171−176.

Khanna, N. C., Helwig, E. D., Ikebuchi, N. W., Fitzpatrick, S., Bajwa, R., & Waisman, D. M. (1990) *Biochemistry 29*, 4852−4862.

Kristensen, T., Saris, C. J., Hunter, T., Hicks, L. J., Noonan, D. J., Glenney, J. R., Jr., & Tack, B. F. (1986) *Biochemistry 25*, 4497−4503.

Kube, E., Weber, K., & Gerke, V. (1991) *Gene 102*, 255−259.

Luecke, H., Chang, B. T., Mailliard, W. S., Schlaepfer, D. D., & Haigler, H. T. (1995) *Nature 378*, 512−515.

MacLaren, D. C., & Clarke, S. (1995) *Protein Expression Purif. 6*, 99−108.

Nakata, T., Sobue, K., & Hirokawa, N. (1990) *J. Cell Biol. 110*, 13−25.

Nigg, E. A., Cooper, J. A., & Hunter, T. (1983) *J. Cell Biol. 96*, 1601−1609.

Osborn, M., Johnsson, N., Wehland, J., & Weber, K. (1988) *Exp. Cell Res. 175*, 81−96.

Powell, M. A., & Glenney, J. R. (1987) *Biochem. J. 247*, 321−328.

Raynal, P., & Pollard, H. B. (1994) *Biochim. Biophys. Acta 1197*, 63−93.

Regnouf, F., Rendon, A., & Pradel, L. A. (1991) *J. Neurochem. 56*, 1985−1996.

Ryan, M. T., Naylor, D. J., Hoogenraad, N. J., & Hoj, P. B. (1995) *J. Biol. Chem. 270*, 22037−22043.

Senda, T., Okabe, T., Matsuda, M., & Fujita, H. (1994) *Cell Tissue Res. 277*, 51−60.
Swairjo, M. A., & Seaton, B. A. (1994) *Annu. Rev. Biophys. Biomol. Struct. 23*, 193−213.
Swairjo, M. A., Concha, N. O., Kaetzel, M. A., Dedman, J. R., & Seaton, B. A. (1995) *Nature, Struct. Biol. 2*, 968−974.
Thiel, C., Weber, K., & Gerke, V. (1991) *J. Biol. Chem. 266*, 14732−14739.
Thiel, C., Osborn, M., & Gerke, V. (1992) *J. Cell Sci. 103*, 733−742.
Urbancikova, M., & Hitchcock-DeGregori, S. E. (1994) *J. Biol. Chem. 269*, 24310−24315.
Waisman, D. M. (1995) *Mol. Cell Biochem. 149/150*, 301−322.
Ward, A., Summers, M. J., & Sim, E. (1995) *Biochem. Pharmacol. 49*, 1759−1767.
Wright, J. F., Kurosky, A., & Wasi, S. (1994) *Biochem. Biophys. Res. Commun. 198*, 983−989.
Xu, G. Q., & Hitchcock-DeGregori, S. E. (1988) *J. Biol. Chem. 263*, 13962−13969.
Yang, Y. F., & Wells, W. W. (1990) *J. Biol. Chem. 265*, 589−593.
Yeatman, T. J., Updyke, T. V., Kaetzel, M. A., Dedman, J. R., & Nicolson, G. L. (1993) *Clin. Exp. Metastasis 11*, 37−44.

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