Modulation of Annexin II Tetramer by Tyrosine Phosphorylation†

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ABSTRACT: Annexin II tetramer (AIIt) is a Ca^{2+}-dependent phospholipid-binding phosphoprotein. In cells either expressing transforming protein tyrosine kinases or treated with growth factors such as PDGF, AIIt has been shown to contain increased levels of phosphotyrosine. Therefore, we have examined the effects of the *in vitro* phosphorylation of AIIt by $pp60^{c\text{-}src}$ on several activities of the protein. AIIt was phosphorylated by $pp60^{c\text{-}src}$ to 0.91 ± 0.07 mol of phosphate/mol of AIIt (mean ± SD). The protein tyrosine phosphorylation of AIIt completely inhibited the ability of the protein to bind to and bundle F-actin. In contrast, the phosphoprotein and native protein bound to purified adrenal medulla chromaffin granules with similar affinity; however, the chromaffin granule bridging activity of the phosphoprotein was abolished. The inhibition of the chromaffin granule bridging activity of the phosphoprotein could be partially reversed by the addition of millimolar Ca^{2+}. Furthermore, the phosphorylation of AIIt by $pp60^{c\text{-}src}$ inhibited the *in vitro* ability of this annexin to form a complex consisting of plasma membrane, chromaffin granules, and AIIt. In addition to binding to biological membranes, some annexin proteins have been shown to possess carbohydrate-binding activity. Although native AIIt bound to a heparin affinity column, tyrosine phosphorylation of AIIt blocked the ability of the protein to bind to the heparin affinity column. These results suggest that the tyrosine phosphorylation of AIIt is a negative modulator of AIIt and that the dephosphorylation of AIIt might be necessary for activation of the protein.

Annexin II tetramer (AIIt)[1] is an abundant Ca^{2+}-binding protein that binds to anionic phospholipids (Glenney, 1985; Powell & Glenney, 1987). AIIt has been localized to the apical plasma membrane (Cooper & Hunter, 1982; Courtneidge *et al.*, 1983; Greenberg & Edelman, 1983a; Nigg *et al.*, 1983; Gerke & Weber, 1984; Drust & Creutz, 1991) and to the extracellular surface of some cells (Tressler & Nicolson, 1992; Tressler *et al.*, 1993). A number of biological properties have been reported for the protein [reviewed in Crompton *et al.* (1988); Klee, 1988; Tokuda *et al.*, 1988; Burgoyne & Geisow, 1989; Gerke, 1989; Geisow *et al.*, 1990; Johnsson *et al.*, 1990; Ikebuchi & Waisman, 1990b; Creutz, 1992; Raynal & Pollard, 1994; Waisman, 1995]. For example, AIIt has been shown to bind to F-actin and promote the formation of anisotropic F-actin bundles. Both the Ca^{2+}-dependence and affinity of binding of AIIt to F-actin have been shown to be physiologically relevant (Gerke & Weber, 1984; Ikebuchi & Waisman, 1990a; Jones *et al.*, 1992). AIIt also binds heparin with a millimolar Ca^{2+} requirement (Waisman *et al.*, 1995). This high Ca^{2+} requirement has suggested that the heparin-binding activity of AIIt may play a role in the function of extracellular AIIt. AIIt has also been demonstrated to bind to and bridge chromaffin granules at micromolar Ca^{2+} concentrations (Drust & Creutz, 1988; Jones *et al.*, 1994).

The physiological function of AIIt has not been established. However, the presence of the protein at the plasma membrane and also its ability to reconstitute secretion in cells previously deprived of it (Ali *et al.*, 1989; Ali & Burgoyne, 1990; Sarafian *et al.*, 1991) have been interpreted to suggest that AIIt may play a role as a "docking" agent which brings secretory granules into sufficiently close apposition with the apical plasma membrane so as to allow fusion of the vesicle to occur [reviewed in Raynal and Pollard (1994); Burgoyne *et al.*, 1993; Creutz, 1992]. Electron microscopic evidence has also suggested the formation of a secretory granule−plasma membrane bridge by AIIt (Nakata *et al.*, 1990; Senda *et al.*, 1994). AIIt has also been suggested to be involved in the endocytotic pathway [Emans *et al.*, 1993; reviewed by Gruenberg and Emans (1993)] and has been proposed to play a role in the membrane trafficking of endocytotic vesicles. In contrast, the presence of AIIt on the external surface of some cells has led to the suggestion that AIIt may play a role in cell−cell adhesion (Tressler & Nicolson, 1992).

AIIt has been shown to comprise two copies of a 36-kDa heavy chain, otherwise called annexin II monomer (AIIm), and two copies of the 11-kDa light chain, p11, (Erikson *et al.*, 1984; Gerke & Weber, 1984). The 36-kDa heavy chain consists of two functional domains. The first, the amino-terminal domain, contains the first 30 amino acids of the amino terminus of the heavy chain, incorporates the phosphorylation sites (Schlaepfer & Haigler, 1988; Weber *et al.*, 1987; Gould *et al.*, 1986; Johnsson *et al.*, 1986a; Glenney & Tack, 1985) and the binding site for the p11 light chain (Johnsson *et al*, 1988; Glenney, *et al.*, 1986; Johnsson *et al.*, 1986b), while the remaining carboxyl domain comprises the sites for Ca^{2+}, phospholipid (Glenney, 1986; Johnsson *et al.*, 1986b), and F-actin binding (Jones *et al.*, 1992; Glenney, *et al.*, 1987). Whereas AIIm is distributed throughout the cell (Osborn *et al.*, 1988; Zokas & Glenney, 1987),

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[1] Abbreviations: EGTA, ethylene glycol bis(b-amino ethyl ether)-*N*,-*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; AIIt, annexin II tetramer; AIIm, annexin II monomer; ATP, adenosine triphosphate; DTT, dithiothreitol.

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the association of the light chain with AIIm appears to mediate the interaction of AIIt with the plasma membrane (Thiel *et al.*, 1992). Thus the formation of the heterotetramer determines the specific cellular localization of this protein.

The amino-terminal domain of AIIt contains phosphorylation sites for both protein serine and tyrosine kinases. Analysis of the phosphorylation of this protein *in vivo* has involved immunoprecipitation with an antibody to the 36-kDa subunit. Therefore, these mixtures contain monomeric and tetrameric forms of annexin II (AII). Activation of protein kinase C in AG1523 fibroblasts or MDBK kidney cells has been shown to result in the phosphorylation of AII at serine-25 (Gould *et al.*, 1986). The *in vitro* phosphorylation of AIIt (Johnstone *et al.*, 1992; Khanna *et al.*, 1986, 1987; Gould *et al.*, 1986) also occurs at serine-25 (Gould *et al.*, 1986). The phosphorylation of AIIt by protein kinase C has been shown to increase the K_d (Ca^{2-}) for aggregation of phospholipid liposomes without affecting the phospholipid-binding properties of the protein (Johnstone *et al.*, 1992).

AIIt has also been shown to be phosphorylated *in vivo* by protein tyrosine kinases. For example, the expression of transforming protein kinases in a variety of cells has been shown to correlate with the appearance of phosphotyrosine in AII (Radke & Martin, 1979; Erikson & Erikson, 1980; Martinez *et al.*, 1982; Amini & Kaji, 1983), and in many cells AII is a major *in vivo* substrate of $pp60^{v\text{-}src}$ (Cooper & Hunter, 1983; Martinez *et al.*, 1982; Greenberg & Edelman, 1983b). AIIt is also a major *in vivo* substrate for the constitutive protein tyrosine kinase activity of bovine articular chondrocytes (Grima *et al.*, 1994). Activation of growth factor receptors has also been shown to result in the protein phosphorylation of AIIt. The treatment of fibroblasts with PDGF results in the tyrosine phosphorylation of AII (Isacke *et al.*, 1986; Brambilla *et al.*, 1991; Zippel *et al.*, 1989). The phosphorylation of AII in $pp60^{v\text{-}src}$-transformed cells or in cells activated by PDGF is identical to the site phosphorylated on the protein *in vitro* by $pp60^{c\text{-}src}$, namely, tyrosine-23 (Isacke *et al.*, 1986).

In the present work, we have examined the consequences of the tyrosine phosphorylation of AIIt on the biological activities of the protein. We report here that, in contrast to native AIIt, $pp60^{c\text{-}src}$-phosphorylated AIIt neither binds to nor bundles F-actin at physiological levels of Ca^{2-}, and even Ca^{2+} levels as high as 1 mM cannot reverse this inhibition. Secondly, the phosphorylation of AIIt by $pp60^{c\text{-}src}$ results in an inhibition of the ability of this protein to aggregate chromaffin granules, while the binding of both native and phosphorylated proteins, to the chromaffin granules, are identical under these conditions. Furthermore, in contrast to the ability of native AIIt to promote the *in vitro* formation of complexes consisting of AIIt-bridged plasma membrane and complexes consisting of chromaffin granules bridged to plasma membrane, phosphorylated AIIt binds to both plasma membrane and chromaffin granules but does not promote the generation of the bridged complexes.

MATERIALS AND METHODS

Phosphorylation of Annexin IIt. AIIt at 60 μg/mL was incubated at 30 °C for 30 min in 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EGTA, 0.6 mM $CaCl_2$, 1.5 mg/mL of baculovirus recombinant $pp60^{c\text{-}src}$, and 100 μg/mL of lipid vesicles which were taken from a stock containing 200 μg of phosphatidylserine/mL, 200 μg of phosphatidylcholine/mL, and 40 μg of diolein/mL. The reaction was initiated by addition of 25 μM ATP (200−2000 cpm/pmol [γ-^{32}P]-ATP. To quantify the stoichiometry of tyrosine phosphorylation of AIIt, 25 μL was removed from the reaction mixture and either precipitated with 25% trichloroacetic acid and 2% sodium pyrophosphate and subjected to scintillation counting or, alternatively, boiled with 1 volume of SDS−PAGE sample buffer (0.25 M Tris−HCl, pH 6.8, 10% SDS, 20% glycerol, 2 mM EGTA, 2 mM EDTA, and 20 mM β-mercaptoethanol) and analyzed by SDS−PAGE. "Sham"-phosphorylated annexin II tetramer was produced by subjecting annexin II tetramer to the phosphorylation reaction in the absence of ATP. Under our assay conditions AIIt was maximally phosphorylated to 0.91 ± 0.07 mol of phosphate/mol of AIIt (mean ± SD, n = 11) by $pp60^{c\text{-}src}$. Tryptic digestion and amino acid sequence analysis of the phosphorylated protein confirmed that, as has been reported for the *in vivo* phosphorylated AIIt (Isacke *et al.*, 1986), tyrosine-25 was the site of phosphorylation for the *in vitro* phosphorylation (data not shown).

Extraction of Phosphorylated Annexin IIt. Annexin II tetramer was phosphorylated as described above in a final volume of 4 μL. After 30 min of incubation, 10 μL was removed to determine the stoichiometry of tyrosine phosphorylation. The reaction mixture was adjusted to a final EGTA concentration of 20 mM (pH 7.5) and 20 mM EDTA (pH 7.5) and was incubated for 15 min at 20 °C. This mixture was centrifuged (14 000g for 30 min) in order to separate the phosphorylated AIIt from the phospholipid vesicles. The supernatant containing the phosphorylated AIIt was adjusted to a final concentration of 50 mM MES (pH 6.0) and loaded onto a 1 mL Fast S column that had been pre-equilibrated with 50 mM MES. The phosphorylated annexin II tetramer was eluted from the column with 0.5 M NaCl in MES (pH 6.0), and the fractions containing AIIt were determined by absorbance at 280 nm (extinction coefficient of 0.65 for 1 mg/mL) and pooled. The pooled fractions were desalted with 300 mM sucrose and 25 mM HEPES (pH 7.5) on a 10 mL PD10 column and stored at −80 °C until used.

AIIt-Dependent Plasma Membrane−Chromaffin Granule Complex Formation. Plasma membrane was isolated and purified from bovine adrenal medulla as per the method of De Block *et al.* (1990). Typically, 0.1 mg of chromaffin granules was incubated in the presence or absence of 1.23 μM AIIt and 0.21 mg of purified plasma membrane in a buffer containing 0.3 M sucrose, 25 mM HEPES (pH 7.5), 20 μM $CaCl_2$, and 30 mM KCl for 15 min at 20 °C. Aliquots (0.6 mL) were top loaded onto a sucrose gradient that contained 25 μM $CaCl_2$ and 25 mM HEPES (pH 7.5) throughout and consisted of 1.8 mL each of 0.31, 0.91, 1.1, 1.25, and 1.6 M sucrose and 1 mL of 2.0 M sucrose. The gradients were centrifuged at 32 500g for 60 min, and then fractions (0.32 mL) were removed by an automated gradient maker (Buchler Auto Densi-Flow IIC) running in reverse mode.

Heparin Affinity Column Chromatography. Heparin affinity chromatography was performed according to (Kojima *et al.*, 1992). Native (0.25 mg) or phosphorylated AIIt (0.025 mg) was equilibrated with buffer containing 25 mM Tris-(pH 7.5), 150 mM NaCl, 1 mM DTT, and 2.5 mM $CaCl_2$

and applied to a 0.4 mL column of heparin−agarose (Sigma). The column was washed with this buffer, and bound protein was eluted with buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 2 mM EDTA.

Miscellaneous Techniques. AIIt was prepared from bovine lung (Khanna *et al.*, 1990) and stored in 50 mM KCl at −70 °C. The protein was essentially homogeneous as determined by SDS−PAGE and contained <0.05 mol of phosphate/mol of AIIt. Chromaffin granules were purified according to Drust and Creutz (1988). Protein concentration was measured with the Bradford (1976) Coomassie Blue dye-binding method using bovine serum albumin as a standard. Alternatively, protein concentrations were determined spectrophotometrically using appropriate extinction coefficients: actin, $A_{290nm} = 0.65$ for 1 mg/mL (Wegner, 1976); annexin IIt, $A_{280nm} = 0.65$ for 1 mg/mL (Gerke & Weber, 1985). EGTA was used to buffer calcium in all solutions, and free calcium levels were calculated according to Fabiato and Fabiato (1979) using the log K_a values of Vianna (1975). F-actin binding and bundling assays were performed by cosedimentation of AIIt with F-actin in bundling buffer (50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT, 0.4 mM $CaCl_2$, 0.2 mM EGTA, and 25 mM MES, pH 6.5) as described by Ikebuchi and Waisman (1990a). F-actin bundling was also assessed by measurement of the light-scattering intensity perpendicular to the incident light in a Perkin-Elmer 650-10S spectrofluorometer as described (Ikebuchi & Waisman, 1990a). Lipid vesicles for the annexin phosphorylation reaction were prepared fresh daily according to Johnstone *et al.* (1992). Human pp60$^{c\text{-}src}$ was made using a baculovirus vector and purified by hydroxylapetite and immunoaffinity chromatography (J. Bjorge and D. J. Fujita, manuscript in preparation).

RESULTS

Bundling of F-Actin by Native and pp60$^{c\text{-}src}$-Phosphorylated AIIt. AIIt has been shown to bind to F-actin (Glenney *et al.*, 1987; Ikebuchi & Waisman, 1990a; Glenney & Glenney, 1985; Gerke & Weber, 1984,1985) with an affinity similar to other F-actin binding proteins [Ikebuchi & Waisman, 1990a; reviewed in Ikebuchi and Waisman (1990b)]. The F-actin binding activity of AIIt is Ca^{2+}-dependent and reversible by removal of Ca^{2+} (Ikebuchi & Waisman, 1990a), and the binding of AIIt to F-actin results in the generation of large anisotropic F-actin bundles. In contrast to the phosphorylation sites of AIIt, which have been shown to exist at the amino terminus of the protein, the F-actin bundling domain of AIIt has been shown to exist near the carboxyl terminal of the protein (Jones *et al.*, 1992).

In order to examine the F-actin bundling activity of the native and phosphorylated protein, F-actin was incubated with either form of AIIt and examined by spectrophotometry or cosedimentation. Analysis of F-actin bundling activity by light scattering suggested that the F-actin bundling activity of AIIt was inhibited by tyrosine phosphorylation (Figure 1). The AIIt was not inactivated during the tyrosine phosphorylation reaction since AIIt, which was incubated with pp60$^{c\text{-}src}$ in the absence of ATP and purified from the kinase (the sham-phosphorylated protein), was fully active (Figure 1). Alternatively, the F-actin bundling activity of AIIt was analyzed by low-speed centrifugation. Under these conditions mainly bundled F-actin and AIIt associated with

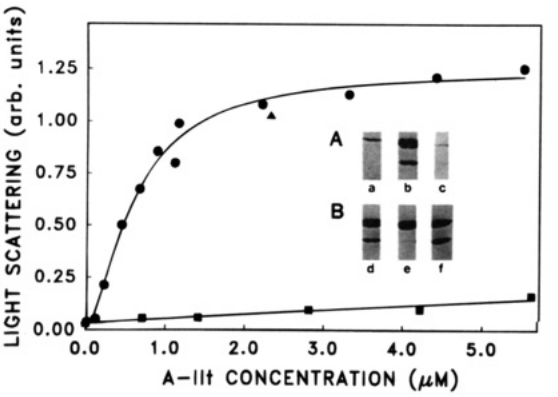

FIGURE 1: Effect of tyrosine phosphorylation of AIIt on its ability to bundle and bind to F-actin. F-actin (2.38 μM) was preincubated for 10 min at 20 °C in a buffer containing 50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT, 0.4 mM $CaCl_2$, 0.2 mM EGTA, and 25 mM MES, pH 6.5. Native AIIt (●), phosphorylated AIIt (■) at the concentrations shown, and sham-phosphorylated AIIt (▲) at 2.31 μM were 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 or low-speed centrifugation (15 600*g*, 10 min; inset A). F-actin binding activity was analyzed by high-speed centrifugation (353 000*g*, 20 min; inset B). In the inset, AIIt is represented by the lower band: a, no added AIIt; b and d, 0.44 μM native AIIt; c and e, 5.6 μM phosphorylated AIIt; f, 2.31 μM sham-phosphorylated AIIt. Results are representative of three independent experiments.

these F-actin bundles will be sedimented. As shown in Figure 1A, native (Figure 1A, lane b) but not phosphorylated AIIt (Figure 1A, lane c) formed F-actin bundles.

The inhibition of the F-actin bundling activity of phosphorylated AIIt presented the possibility that tyrosine phosphorylation of AIIt by pp60$^{c\text{-}src}$ blocked the F-actin bundling activity of AIIt without affecting the binding of AIIt to F-actin. Alternatively, since the bundling of F-actin by AIIt first requires the binding of the protein to F-actin, it was also possible that the phosphorylated protein could not bind to F-actin and therefore F-actin bundling could not occur. When the F-actin binding activity of AIIt was examined by high-speed centrifugation, it was observed that the phosphorylated protein did not bind to and cosediment with F-actin (Figure 1B, lane e). These results therefore suggested that phosphorylation of AIIt by pp60$^{c\text{-}src}$ inhibited the ability of AIIt to bind to F-actin.

Ca^{2+}-Dependence of AIIt Binding and Bundling of F-Actin. Since the binding and bundling of F-actin by AIIt is Ca^{2+}-dependent (Ikebuchi & Waisman, 1990b), we investigated the effect of tyrosine phosphorylation on the Ca^{2+}-dependence of AIIt-mediated F-actin binding and bundling. When the F-actin bundling activity of native and phosphorylated AIIt was examined by light scattering (Figure 2), it was observed that the bundling activity of the native but not the phosphorylated protein was activated by Ca^{2+}. We also examined the F-actin bundling activity of native and phosphorylated AIIt by low-speed centrifugation (Figure 2A). Consistent with the light-scattering data, it was observed that elevated concentrations of Ca^{2+} could not reverse the inhibitory effects of tyrosine phosphorylation on the F-actin bundling activity of AIIt. The inability of phosphorylated AIIt to bundle F-actin was due to the inability of phosphorylated AIIt to bind to F-actin, and, even in the presence of 1 mM Ca^{2+}, the phosphorylated AIIt did not bind to F-actin (Figure 2B). These results therefore suggested that tyrosine

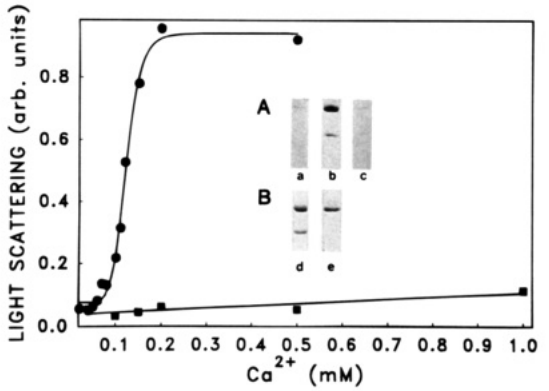

FIGURE 2: Ca^{2+}-dependence of F-actin bundling by native and phosphorylated AIIt. F-actin (2.38 μM) was preincubated for 10 min at 20 °C in a buffer containing 50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT, 0.4 mM $CaCl_2$, 0.2 mM EGTA, and 25 mM MES, pH 6.5. Native (●) and phosphorylated AIIt (■), both at 1.4 μM, were added to the reaction mixture, with sufficient Ca^{2+} to produce the final Ca^{2+} values shown, followed by a further incubation for 10 min at 20 °C. The aliquots were analyzed for F-actin bundling activity by light scattering or low-speed centrifugation (15 600*g*, 10 min; inset A). F-actin binding activity was analyzed by high-speed centrifugation (353 000*g*, 20 min; inset B). In the inset, AIIt is represented by the lower band: a, native AIIt in the absence of added Ca^{2+} [$Ca^{2+}_f = 10^{-8}$M, by Fabiato and Fabiato (1979)]; b, native AIIt in 150 μM Ca^{2+}; c, phosphorylated AIIt in 1 mM Ca^{2+}; d, native AIIt in 100 μM Ca^{2+}; e, native AIIt in 1 mM Ca^{2+}. Results are representative of three independent experiments.

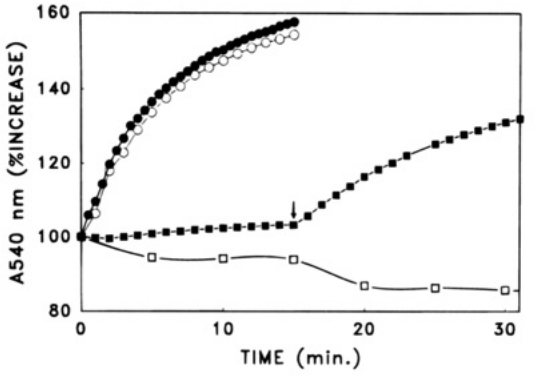

FIGURE 3: Inhibition of chromaffin granule bridging by phosphorylated annexin IIt. Chromaffin granules were purified as described in Materials and Methods and were then incubated with 20 μM $CaCl_2$, 50 mM KCl, 25 mM Hepes (pH 7.5), and sucrose to a final concentration of 340 mOsmol. Immediately after taking the first reading of optical density, 2 μM phosphorylated AIIt (■) or 2 μM native AIIt (●) or 2 μM sham-phosphorylated AIIt (○) or an equal volume of 300 mM sucrose, 25 mM Hepes (pH 7.5) (□) was added, bringing the aliquot volume to 500 μL. Absorbance was read at the times shown. $CaCl_2$, to a final concentration of 2 mM, was added to both samples at the arrow. Changes in optical absorbance are expressed as a percentage of starting optical density (no added AIIt) determined spectrophotometrically at a wavelength of 540 nm (see Materials and Methods). Data shown are representative of three independent experiments.

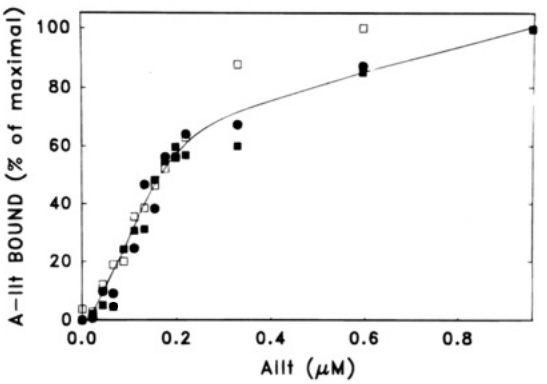

FIGURE 4: Binding of $pp60^{c\text{-}src}$-phosphorylated AIIt and native AIIt to isolated chromaffin granules. Chromaffin granules were purified, and the $pp60^{c\text{-}src}$-phosphorylated AIIt was prepared as described in Materials and Methods. Chromaffin granules were incubated, at 20 °C, in a buffer containing 50 mM KCl, 50 μM Ca^{2+}, 0.3 M sucrose, 25 mM Hepes (pH 7.5), and the various concentrations of phosphorylated (■) and native AIIt (●) shown, to a final volume of 500 μL. Aliquots were centrifuged (15 600*g*, 15 min), the ^{32}P content of the phosphorylated AIIt-granule pellet complex was counted without scintillant (□), and then all pellets were resuspended in SDS buffer. After SDS–PAGE, protein bands were visualized with Coomassie Brilliant Blue (R-250), and AIIt bands thus obtained were cut from the gel and quantified spectrophotometrically as per Materials and Methods. Data shown are representative of three independent experiments.

phosphorylation of AIIt by $pp60^{c\text{-}src}$ completely blocked the interaction of AIIt with F-actin.

Bridging of Chromaffin Granules by Native and $pp60^{c\text{-}src}$-Phosphorylated AIIt. The interaction of AIIt with biological membranes has been studied with chromaffin granule membranes serving as the model system. AIIt has been demonstrated to mediate the Ca^{2+}-dependent aggregation (bridging) of chromaffin granules, half-maximal bridging occurs at about 2 μM Ca^{2+} (Drust & Creutz, 1988). Consistent with that report we found that the addition of AIIt to chromaffin granules, in the presence of 20 μM Ca^{2+}, resulted in a dramatic bridging of granules, which was maximal within 30 min (Figure 3). Similarly, sham-phosphorylated AIIt, under the same conditions, shows similar kinetics of bridging. In contrast, phosphorylated AIIt, again in the presence of 20 μM Ca^{2+}, did not activate chromaffin granule bridging. The inhibition of the chromaffin granule bridging activity of tyrosine phosphorylated AIIt could, however, be partially reversed by addition of millimolar Ca^{2+} (Figure 3) or by the addition of excess native AIIt (data not shown).

Binding of Native and $pp60^{c\text{-}src}$-Phosphorylated AIIt to Chromaffin Granules. In order to examine if the loss in chromaffin granule bridging activity of phosphorylated AIIt corresponded with a loss in chromaffin granule binding activity, native or phosphorylated AIIt was incubated with chromaffin granules and the AIIt content of the pelleted granules was examined. Figure 4 presents the dose-dependency of binding of AIIt to chromaffin granules. Perhaps surprisingly, the native and phosphorylated protein bound to chromaffin granules with similar affinity, suggesting the inhibition of chromaffin granule bridging activity of AIIt by tyrosine phosphorylation was not due to a disruption of the binding of AIIt to chromaffin granules.

Modulation of the Plasma Membrane–AIIt–Chromaffin Granule Complex. Electron microscopic evidence (Nakata *et al.*, 1990; Senda *et al.*, 1994) has suggested that AIIt may form a bridge between the cytosolic face of the plasma membrane and secretory granules. Furthermore, AIIt has also been shown to participate in the binding of the plasma membrane of RAW117 to the plasma membrane of endothelial cells (Tressler & Nicolson, 1992; Tressler *et al.*, 1993). These results therefore suggest that AIIt possesses the ability to form complexes, *in vivo*, consisting of bridged plasma membrane or complexes consisting of chromaffin granules bridged to plasma membrane.

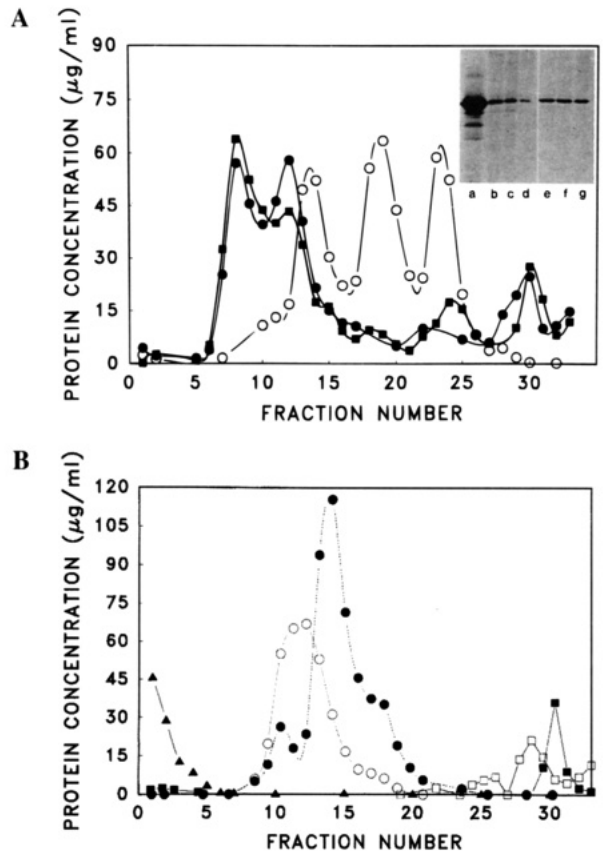

FIGURE 5: Formation of a plasma membrane−AIIt−chromaffin granule complex by native but not phosphorylated AIIt. Typically, 0.1 mg of chromaffin granules was incubated in the presence or absence of 1.23 μM AIIt and 0.21 mg of purified plasma membrane in a buffer containing 0.3 M sucrose, 25 mM HEPES (pH 7.5), 20 μM $CaCl_2$, and 30 mM KCl for 15 min at 20 °C. Aliquots (0.6 mL) were top loaded onto a sucrose gradient (methods), and fractions (0.32 mL) were removed by an automated gradient maker (Buchler Auto Densi-Flow IIC) running in reverse mode. A. Standard reactions were conducted in the presence of 1.23 μM phosphorylated (●) or native AIIt (○) or in the absence (■) of AIIt. In the inset, fractions from the sucrose gradient analysis of reaction mixtures conducted in the presence of native (lanes b−d) or phosphorylated AIIt (lanes e−g) were pooled and analyzed by SDS−PAGE (lanes b−d) and autoradiography (lanes e−g): a, AIIt standard; b, fractions 13−16; c, fractions 17−21; d, fractions 22−25; e, fractions 7−10; f, fractions 11−15; g, fractions 29−31. B. The reaction mixture contained 0.3 M sucrose, 25 mM HEPES (pH 7.5), 20 μM $CaCl_2$, 30 mM KCl, and either 1.23 μM AIIt (▲) or 0.21 mg of plasma membrane/mL (○), 1.23 μM AIIt and 0.21 mg of plasma membrane/mL (●), 0.1 mg of chromaffin granules/mL (□), or 1.23 μM AIIt and 0.1 mg of chromaffin granules/mL (■).

In order to investigate the possible formation of AIIt-dependent bridges between plasma membranes and chromaffin granules, *in vitro*, we incubated purified plasma membrane, AIIt, and chromaffin granules in the presence of Ca^{2+} and separated the mixture on a sucrose gradient. As shown in Figure 5A, when plasma membrane, AIIt, and chromaffin granules were incubated in the presence of Ca^{2+} and the mixture was separated on a sucrose gradient, three protein peaks in fractions 13 and 14, fraction 19, and fraction 23 were resolved. SDS−PAGE analysis suggested the presence of AIIt in all three peak fractions (Figure 5A, lanes b−d). Furthermore, as shown in Figure 5B, sucrose gradient analysis of mixtures of composed of plasma membrane and AIIt or chromaffin granules and AIIt, suggested that complexes consisting of AIIt-bridged plasma membrane were eluted at fraction 14 (Figure 5B) and that complexes consisting of AIIt-bridged chromaffin granules were eluted at fraction 30 (Figure 5B). Furthermore, direct analysis of peak fractions for plasma membrane (5′-nucleotidase activity) and chromaffin granules (catecholamine content) suggested that peak fractions 19 and 23 contained both plasma membrane and chromaffin granules (Fitzpatrick *et al.*, manuscript in preparation). The formation of peak fractions 19 and 23 was Ca^{2+}-dependent and specific to AIIt, since annexin I, annexin V, or annexin VI did not cause the formation of those peak fractions (data not shown). These results suggest that the incubation of AIIt with plasma membrane and chromaffin granules results in the formation of complexes of AIIt-bridged plasma membrane (Figure 5A, fraction 14) and AIIt-dependent complexes of chromaffin granules bridged to plasma membrane (Figure 5A, fractions 19 and 23).

However, when phosphorylated AIIt was incubated with plasma membrane and chromaffin granules in the presence of Ca^{2+} followed by analysis on sucrose gradients, the pattern of protein peaks was not similar to the pattern produced by incubation of plasma membrane and chromaffin granules with native AIIt. The pattern of protein peaks resolved after centrifugation of a mixture of plasma membrane, chromaffin granules, and phosphorylated AIIt was identical to the pattern of protein peaks produced by sucrose gradient analysis of a mixture of plasma membrane and chromaffin granules in the absence of AIIt. SDS−PAGE analysis of the protein peaks resolved from the reaction mixture containing plasma membrane, phosphorylated AIIt, and chromaffin granules (Figure 5A, lanes e−g) suggested that phosphorylated AIIt bound to the plasma membrane and chromaffin granule fractions. These results therefore suggest that phosphorylated AIIt cannot form bridged complexes of plasma membrane or complexes consisting of chromaffin granules bridged to plasma membrane.

Carbohydrate-Binding Activity of AIIt. Recently, annexin IV (AIV) was shown to exhibit Ca^{2+}-dependent carbohydrate-binding activity (Kojima *et al.*, 1992). The carbohydrate-binding activity of AIV was measured with solid-phase assays using biotin-labeled fetuin and horseradish peroxidase-labeled heparin as ligands. Furthermore, AIV was also shown to bind to a heparin affinity column in a Ca^{2+}-dependent manner. We therefore examined the possible carbohydrate-binding activity of AIIt by heparin affinity chromatography. As shown in Figure 6, native AIIt binds to the heparin affinity column in the presence of millimolar Ca^{2+} and was eluted from the column by buffer containing EDTA. In contrast, when phosphorylated AIIt was applied to the heparin affinity column, the protein did not bind and was recovered in the column flow-through. These results suggest that the carbohydrate-binding activity of AIIt is also inhibited by tyrosine phosphorylation.

Annexin II has been shown to be phosphorylated on tyrosine residues in cells expressing transforming tyrosine kinases or in cells treated with growth factors such as PDGF which activate tyrosine kinase receptors. The site of phosphorylation of annexin II has been identified at the N-terminal domain, Tyr-23 (Isacke *et al.*, 1986).

In vitro, annexin II tetramer (AIIt) can be phosphorylated by pp60$^{c\text{-}src}$, and the site for the *in vitro* phosphorylation is also Tyr-23. Under our assay conditions AIIt was maximally phosphorylated to 0.91 ± 0.07 mol of phosphate/mol of AIIt (mean ± SD, n = 11) by pp60$^{c\text{-}src}$. Gel permeation

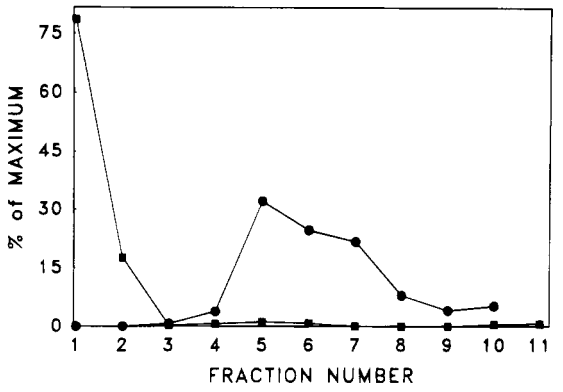

FIGURE 6: Phosphorylation inhibits the carbohydrate-binding activity of AIIt. Native (0.25 mg) (●) or pp60$^{c\text{-}src}$-phosphorylated AIIt (0.025 mg) (■) was applied to a 0.40 mL heparin−agarose column equilibrated with 25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 2.5 mM $CaCl_2$. A single 4.0 mL fraction was collected (fraction 1). The column was washed with this buffer (which was collected as a single 4.0 mL fraction; fraction 2), and bound proteins were eluted with 25 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 2 mM EDTA and collected as a series of 0.5 mL fractions.

chromatography of AIIt suggested that the tyrosine phosphorylation of the protein did not cause the dissociation of the tetramer (data not shown). The observation that application of the tyrosine-phosphorylated AIIt to a heparin affinity did not result in the elution of unphosphorylated AIIt (Figure 6) suggests that all the AIIt in the reaction mixture is tyrosine phosphorylated. It is therefore possible that the formation of the annexin II tetramer may result in the exposure of the tyrosine phosphorylation site of only one of the two 36-kDa subunits. Alternatively, it is possible that the tyrosine phosphorylation of the tetramer results in a conformational change which blocks further access to the second tyrosine phosphorylation site.

Since the phosphorylation of AIIt on tyrosine has been suggested to be an important regulatory event, we examined its effects on several activities of the protein. The activities of AIIt that were examined include Ca^{2+}-dependent F-actin bundling activity, chromaffin granule bridging activity, and AIIt-dependent formation of complexes consisting of AIIt-bridged plasma membrane and complexes consisting of chromaffin granules bridged to plasma membrane.

DISCUSSION

AIIt has been shown to bind to F-actin and promote the formation of large anisotropic F-actin bundles [reviewed in Ikebuchi and Waisman (1990b) and in Waisman (1995)]. The F-actin bundling activity of AIIt is Ca^{2+}-dependent, and the affinity and stoichiometry of F-actin binding are similar to that of other F-actin bundling proteins (Ikebuchi & Waisman, 1990a). Recently, we demonstrated that a nonapeptide to residues 268−294 of AIIt completely inhibited the Ca^{2+}-dependent bundling activity of AIIt (Jones *et al.*, 1992) but did not affect the F-actin binding activity of AIIt. These results established that the F-actin bundling domain resided in the carboxyl-terminal domain of the protein. Since the nonapeptide blocked the F-actin bundling activity of AIIt but did not effect the F-actin binding activity of AIIt, these results also suggested that distinct domains of AIIt were involved in the F-actin binding and bundling properties of AIIt. Furthermore, since the nonapeptide did not inhibit the chromaffin granule bridging activity of AIIt, the domains of AIIt involved in chromaffin granule bridging and F-actin bundling must also be distinct. Our current results indicate that the F-actin binding activity of AIIt is blocked by the tyrosine phosphorylation of the protein (Figures 1 and 2). The inhibition of AIIt-dependent F-actin bundling by the phosphorylated protein is due to the loss of F-actin binding activity of the protein.

In contrast, although phosphorylated AIIt can bind to chromaffin granules with similar affinity as the native protein, the phosphorylated protein shows attenuated chromaffin granule bridging activity. The attenuation of chromaffin granule bridging activity could be partially recovered in the presence of millimolar Ca^{2+}. These results therefore suggest that the chromaffin granule bridging activity and chromaffin granule binding activity of AIIt may also reside in distinct domains of the protein and that only the chromaffin granule bridging activity of the protein is regulated by tyrosine phosphorylation. If it is assumed that the binding of AIIt to chromaffin granules is indicative of the binding of AIIt to other biological membranes, then our results would predict that the phosphorylation of AIIt by pp60^{src} *in vivo* would not affect the binding of the protein to the plasma membrane. In fact, it has been observed that the subcellular localization of AIIt, at the plasma membrane, is not effected by transformation of cells with pp60$^{v\text{-}src}$ (Zokas & Glenney, 1987).

Results from several laboratories have suggested that AIIt can reconstitute exocytosis in detergent-permeabilized cells (Ali & Burgoyne, 1989, 1990; Sarafian *et al.*, 1991). Since AIIt does not possess fusogenic activity but can aggregate biological membranes (Drust & Creutz, 1988), it is reasonable to propose that the stimulation of exocytosis by AIIt is due to the formation of a bridge between the chromaffin granules and plasma membrane. Experimental evidence for this possibility has been observed by electron microscopy, and AIIt has been shown to provide links between plasma membrane and secretory granules (Nakata *et al.*, 1990).

When plasma membrane, AIIt, and chromaffin granules are incubated together and the mixture is resolved on a sucrose gradient, three protein peaks can be resolved that are not observed in the absence of AIIt. Two of the protein peaks, eluting at fractions 19 and 23 (Figure 5A), move to positions on the sucrose gradient distinct from the position on the gradient of complexes of AIIt-bridged plasma membrane or AIIt-bridged chromaffin granules (Figure 5B). Marker enzyme studies have suggested that both peaks contain plasma membrane and chromaffin granules (Fitzpatrick *et al.*, manuscript in preparation). The peak of protein located at fraction 14 of the sucrose gradient (Figure 5A) corresponds to the observed peak of AIIt-bridged plasma membrane (Figure 5B). These results therefore suggest that when mixtures of plasma membrane, chromaffin granules, and AIIt are incubated in the presence of Ca^{2+}, complexes of AIIt-bridged plasma membrane and complexes consisting of chromaffin granules bridged to plasma membrane are formed. Whether or not the complex consisting of chromaffin granules bridged to plasma membrane is structurally similar to the *in vivo* complex remains to be established. It is also unclear why a complex of AIIt-bridged chromaffin granules is not formed and detected on the sucrose gradient.

However, when a mixture of plasma membrane, chromaffin granules, and phosphorylated AIIt is resolved on a

sucrose gradient, the pattern of protein peaks is similar to the pattern of protein peaks observed when chromaffin granules and plasma membrane are incubated in the absence of AIIt. Furthermore, protein peaks corresponding to either the position of the complex consisting of chromaffin granules bridged to plasma membrane or the AIIt-bridged plasma membrane are absent. In contrast, both native and phosphorylated AIIt appear to bind to the plasma membrane and chromaffin granules (Figure 5A). Collectively our results suggest that the native, but not the phosphorylated AIIt, is capable of the bridging of chromaffin granules (Figure 3), formation of a bridged plasma membrane (Figure 5B), or the formation of a complex consisting of chromaffin granules bridged to plasma membrane (Figure 5A).

Little is known about the domains of AIIt involved in the interaction of AIIt with chromaffin granule membranes or plasma membranes, and it is unclear if the binding of biological membranes by AIIt is mediated by the phospholipid binding domains of the protein or requires both phospholipid-binding domains and domains capable of binding membrane proteins. However, considering that the binding of AIIt to these biological membranes is maximal at less than 25 μM Ca^{2+} compared to the substantially higher Ca^{2+} requirement for F-actin binding and heparin binding, it is reasonable to suspect that the domain(s) of AIIt responsible for binding to membranes is (are) distinct from the F-actin- and heparin-binding domains of AIIt. This speculation is further supported by our observation that tyrosine phosphorylation of AIIt inhibits both heparin binding and F-actin binding but not the binding of AIIt to biological membranes.

Recent studies from our laboratory have suggested that, in the absence of added salt, the ability of AIIt to aggregate chromaffin granules is blocked whereas AIIt binding to the granules is maximal (Jones *et al.*, 1994). However, if the mechanism of bridging of chromaffin granules by AIIt involved two distinct membrane-binding domains on a single AIIt molecule, one would predict that the loss of chromaffin granule bridging activity at low salt concentration would be due to a salt requirement for the activation of the second membrane-binding domain of AIIt. We therefore suggested that bridging of chromaffin granules by AIIt could be caused by the interaction of AIIt molecules that were bound to the chromaffin granules and that this AIIt−AIIt interaction was modulated by the salt concentration. It therefore follows from this proposal that the AIIt-dependent formation of aggregates of plasma membrane or the formation of complex consisting of chromaffin granules bridged to the plasma membrane may also be mediated by intermolecular interactions between membrane bound AIIt. It is therefore likely that the domain of AIIt involved in this interaction will be distinct from the domain(s) of the protein involved in membrane binding. If this theory is correct, then it is reasonable to propose that tyrosine phosphorylation of AIIt does not block the membrane-binding domain of AIIt (Figures 4 and 5A) but blocks the intermolecular interactions occurring between membrane-bound AIIt.

Recently, Kojima *et al.* (1992) demonstrated that annexin IV displayed Ca^{2+}-dependent carbohydrate-binding activity. The role that Ca^{2+}-dependent carbohydrate-binding activity plays in the functioning of the protein is unclear. We have found that AIIt also possess Ca^{2+}-dependent carbohydrate-binding activity. Preliminary experiments have suggested that this binding activity requires at least 1 mM $CaCl_2$ (data not shown). Therefore, the millimolar Ca^{2+} requirement of carbohydrate-binding activity of AIIt contrasts with the micromolar Ca^{2+} requirement of the binding of AIIt with chromaffin granules. This presents the possibility that distinct domains of AIIt are involved in the binding to biological membranes and in the binding to heparin. Alternatively, it is possible that heparin and chromaffin granules bind to a similar site on AIIt but do so with different affinities of binding. If the chromaffin granule binding site of AIIt was of higher affinity than the heparin-binding site, then it is possible that this higher affinity ligand produces a greater increase in Ca^{2+} affinity than the lower affinity ligand. Considering that the intracellular Ca^{2+} concentration never reaches millimolar concentrations, it is extremely unlikely that the carbohydrate-binding property of AIIt contributes to any intracellular physiological function of the protein. However, considering the recent reports describing the presence of AIIt on the outside of the cell (Tressler *et al.*, 1993) where the protein is thought to be involved in cell−cell adhesion, it is possible that the carbohydrate-binding activity of AIIt may play a role in the physiological function of extracellular AIIt. The results presented in Figure 6 suggest that the carbohydrate-binding activity of AIIt is blocked by the tyrosine phosphorylation of the protein. It therefore appears that tyrosine phosphorylation of AIIt affects all of the major biological activities of the protein.

The regulation of annexin II appears to involve two distinct and opposite mechanisms. First, the binding of the light chain appears to direct the AII monomer to the plasma membrane (Thiel *et al.*, 1992) and to decrease the Ca^{2+} requirement for bridging of chromaffin granules (Drust & Creutz, 1988) and phospholipid binding and bridging (Powell & Glenney, 1987). In contrast, AIIt is negatively modulated by protein tyrosine phosphorylation, and phosphorylation of the protein by pp60$^{c\text{-}src}$ attenuates both F-actin binding and chromaffin granule bridging. Tyrosine phosphorylation of AIIt also prevents formation of a plasma membrane−AIIt−chromaffin granule complex *in vitro*. It is therefore possible that dephosphorylation of AIIt may represent a positive modulatory signal for the protein.

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