Tyrosine Phosphorylation of Annexin II Tetramer Is Stimulated by Membrane Binding*

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In the present article we have examined if the interaction of the Ca$^{2+}$-binding protein, annexin II tetramer (AIIt) with the plasma membrane phospholipids or with the submembranous cytoskeleton, affects the accessibility of the tyrosine phosphorylation site of AIIt. In the presence of Ca$^{2+}$, pp60$^{c-src}$ catalyzed the incorporation of 0.22 ± 0.05 mol of phosphate/mol of AIIt (mean ± S.D., n = 5). The Ca$^{2+}$-dependent binding of AIIt to purified plasma membrane or phosphatidylserine vesicles stimulated the pp60$^{c-src}$-dependent phosphorylation of AIIt to 0.62 ± 0.04 mol of phosphate/mol of AIIt (mean ± S.D., n = 5) or 0.93 ± 0.07 mol of phosphate/mol of AIIt (mean ± S.D., n = 5), respectively. Phosphatidylserine- or phosphatidylinositol-containing vesicles but not vesicles composed of phosphatidylcholine or phosphatidylethanolamine, stimulated the phosphorylation of AIIt. In contrast, the binding of AIIt to F-actin resulted in the incorporation of only 0.04 ± 0.04 mol of phosphate/mol of AIIt (mean ± S.D., n = 5). These results suggest that the interaction of AIIt with plasma membrane and not the submembranous cytoskeleton, activates the tyrosine phosphorylation of AIIt by inducing a conformational change in the protein resulting in the enhanced exposure or accessibility of the tyrosine-phosphorylation site.

The annexins (reviewed in Refs. 1–5) are a family of Ca$^{2+}$-binding proteins that bind acidic phospholipids. Members of this family contain a region of amino acid homology called the annexin fold (6). Annexin II tetramer (AIIt)$^1$ is an abundant annexin which is composed of two M, 36,000 annexin II subunits and two M, 11,000 subunits. The 36-kDa subunit consists of two functional domains. The first, the amino-terminal regulatory domain contains the first 30 amino acids of the amino terminus of the heavy chain, incorporates the serine and tyrosine-phosphorylation sites and the binding site for the p11 light chain. The remaining carboxyl domain, comprises the binding sites for Ca$^{2+}$, phospholipid, and F-actin (reviewed in Ref. 2). AIIt is associated with the cytosolic surface of the plasma membrane in association with the submembranous cytoskeleton of many secretory cells where the protein has been shown to form links between the plasma membrane and secretory granules (6, 7). Although the exact physiological function of AIIt is unclear, the protein is thought to play a role in Ca$^{2+}$-dependent exocytosis or endocytosis (2).

AIIt has been shown to be phosphorylated in vivo by protein tyrosine kinases. For example, the expression of transforming protein tyrosine kinases in a variety of cells has been shown to correlate with the appearance of phosphotyrosine in AIIt (8, 9) and in many cells AIIt is a major in vivo substrate of pp60$^{c-src}$ (10–12). Activation of transmembrane protein kinase receptors, such as the platelet-derived growth factor receptor, has also been shown to result in the tyrosine phosphorylation of AIIt (13–15). The phosphorylation of AIIt in pp60$^{c-src}$-transformed cells or in cells activated by platelet-derived growth factor factor is identical to the site phosphorylated on the protein in vitro by pp60$^{c-src}$, namely tyrosine 23 (13).

In previous work, we examined the consequences of the tyrosine phosphorylation of AIIt on the biological activities of the protein (16). We reported that pp60$^{c-src}$-phosphorylated AIIt did not bind to heparin or bind to or bundle F-actin. Furthermore, native AIIt but not tyrosine-phosphorylated AIIt could promote the formation of a plasma membrane-AIIt-chromaffin granule complex in vitro (16). We therefore concluded that the tyrosine phosphorylation of AIIt was an inhibitory signal. Consistent with these results, we have shown that activation of adrenal chromaffin cells, with acetylcholine, results in the dephosphorylation of AIIt concomitant with the release of catecholamine (2).

In the present article we have examined the kinetics of phosphorylation of AIIt by pp60$^{c-src}$. The results suggest that the binding of AIIt to the phospholipid component of the plasma membrane stimulates the phosphorylation of the protein by pp60$^{c-src}$.

MATERIALS AND METHODS

Phosphorylation of Annexin II Tetramer—AIIt phosphorylation reactions were performed according to Ref. 16. AIIt at a final concentration of 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 μg/ml baculovirus produced human recombinant pp60$^{c-src}$, and 100 μM lipid vesicles which were taken from a stock containing 200 μg/ml phosphatidylserine, 200 μg/ml phosphatidylcholine, and 40 μg/ml diolein. When incubations were performed in the presence of plasma membrane, 0.5 mM orthovanadate and 3 mg/ml para-nitrophenyl phosphate were included as phosphatase inhibitors. The reaction was initiated by addition of 25 μM ATP (200–2000 cpm/μmol [γ-$^32$P]ATP). To quantify the stoichiometry of 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 % Triton HCl, pH 6.8, 10% SDS, 20% glycerol, 2 μl EGTA, 2 μl EDTA, 20 μM β-mercaptoethanol) and analyzed by SDS-PAGE (17). For experiments testing the phospholipid specificity of phosphorylation (Table II), phospholipid liposomes were prepared by hydrating 4 mg of phospholipid (previously dried from a chloroform solution under N$_2$) with 50 μl HEPES (pH 7.5). The suspensions were mixed in a vortex and sonicated three times for 15 s. Since phosphatidylethanolamine did not

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1 The abbreviations used are: AIIt, annexin II tetramer; PAGE, polyacrylamide gel electrophoresis.
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**TABLE I**

| Addition  | Peptide | Annexin II tetramer |
|-----------|---------|---------------------|
|           | pmol/min | mol phosphate/mol AIIt/min |
| None      | 2.23 ± 0.15 | 0.0037 ± 0.0003 |
| 0.6 mM CaCl<sub>2</sub> | 1.50 ± 0.10 | 0.0083 ± 0.0011 |
| Phospholipid | 1.83 ± 0.31 | 0.048 ± 0.001 |
| 0.6 mM CaCl<sub>2</sub> and phospholipid | 1.77 ± 0.15 | 0.067 ± 0.006 |

Phosphorylation reactions were conducted at 30°C in the presence of buffer A (25 mM HEPES [pH 7.5], 10 mM MgCl<sub>2</sub>, and 0.5 mM EDTA). Peptide (KLSELGHDSTPPSSAYGVSAYKAYT) concentration was 30 μM F-actin concentration was 1 μM. Results are expressed as mean ± S.D. n = 5.

**RESULTS**

**Time Course of Phosphorylation of AIIt by pp60<sup>src</sup>**—Fig. 1 presents the time course of phosphorylation of AIIt by pp60<sup>src</sup>. At low Ca<sup>2+</sup> concentration (0.1 μM) a slow rate of incorporation of phosphate into AIIt was observed with about 0.09 ± 0.08 mol of phosphate/mol of AIIt (mean ± S.D., n = 5) incorporated by pp60<sup>src</sup>-AIIt in 60 min (Fig. 1). When 100 μM Ca<sup>2+</sup> was added to the reaction, the rate of phosphorylation of AIIt by pp60<sup>src</sup> was also slow and after 60 min only 0.22 ± 0.05 mol of phosphate/mol of AIIt (mean ± S.D., n = 5) was incorporated. Increasing the Ca<sup>2+</sup> concentration did not alter the phosphorylation rate and at 1 mM Ca<sup>2+</sup> about 0.21 ± 0.01 mol of phosphate/mol of AIIt (mean ± S.D., n = 5) was incorporated. The addition of phospholipid vesicles to the reaction mixture stimulated the rate of phosphorylation of AIIt and at equilibrium, 0.84 ± 0.05 mol of phosphate/mol of AIIt (mean ± S.D., n = 5) was incorporated. In the presence of 100 μM Ca<sup>2+</sup> and phospholipid vesicles, the phosphorylation of AIIt was very rapid and at equilibrium about 0.93 ± 0.07 mol of phosphate/mol of AIIt (mean ± S.D., n = 9) was incorporated.

The results presented in Fig. 1 suggested that the addition of phospholipid vesicles to the reaction mixture stimulated the rate and extent of phosphorylation of AIIt by pp60<sup>src</sup>. In order to determine if the stimulation of AIIt phosphorylation was due to the interaction of phospholipid vesicles with AIIt or pp60<sup>src</sup>, the effect of phospholipid vesicles on pp60<sup>src</sup>-AIIt activity was examined. The pp60<sup>src</sup>-AIIt activity in these experiments was measured using a peptide to the phosphorylation site peptide in the absence of plasma membrane. The phosphorylation rate at equilibrium 0.93 ± 0.07 mol of phosphate/mol of AIIt (mean ± S.D., n = 9) was incorporated.

**Stimulation of pp60<sup>src</sup>-dependent Phosphorylation of AIIt by Plasma Membrane**—As shown in Fig. 2, at equilibrium, about 0.62 ± 0.04 mol of phosphate (mean ± S.D., n = 5) were incorporated into AIIt by pp60<sup>src</sup> in the presence of purified adrenal medulla plasma membrane (Fig. 2, inset). This compared to 0.22 ± 0.05 mol of phosphate/mol of AIIt (mean ± S.D., n = 5) incorporated in the absence of plasma membrane. The

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stimulation of the phosphorylation of the pp60<sup>src</sup>-dependent phosphorylation of AIIt by plasma membrane could be due to the interaction of AIIt with phospholipolipid or F-actin components of the plasma membrane. However, as shown in Fig. 2, the interaction of AIIt with F-actin did not activate the phosphorylation of AIIt by pp60<sup>src</sup>. In contrast, the interaction of AIIt with phospholipolipid stimulated the rate (Table I) and extent (Fig. 2) of phosphorylation of AIIt. These results therefore suggest that the binding of AIIt to the phospholipolipid component of the plasma membrane is responsible for stimulation of the phosphorylation of AIIt.

**Phosphorylation of AIIt by Partially Proteolyzed pp60<sup>src</sup>—** Both AIIt and pp60<sup>src</sup> are membrane-associated proteins that are known to bind acidic phospholipolipids (23–26). Therefore, it was possible that the phospholipolipid-dependent activation of phosphorylation of AIIt could be due to recruitment of AIIt and pp60<sup>src</sup> to the phospholipolipid vesicles, thereby increasing the proximity of pp60<sup>src</sup> and AIIt. This would result in an increase in the effective concentration of AIIt and pp60<sup>src</sup> in the phosphorylation reaction and could produce an enhanced phosphorylation rate. To examine this possibility, we prepared a partially proteolyzed pp60<sup>src</sup>. The partial proteolysis of the pp60<sup>src</sup> resulted in the loss of a portion of the NH<sub>2</sub>-terminal domain of the enzyme and the loss of the NH<sub>2</sub>-terminal myristic acid. It was therefore expected that the loss of this domain of pp60<sup>src</sup> would inhibit the binding of the enzyme to phospholipolipid liposomes. Therefore, native and partially proteolyzed pp60<sup>src</sup> were incubated with phosphatidylerine liposomes and following centrifugation, the pellets were examined for pp60<sup>src</sup>-activity. As shown in Fig. 3, the partially proteolyzed pp60<sup>src</sup> did not bind to phosphatidylerine liposomes. In contrast, the native pp60<sup>src</sup> bound to the phospholipolipid liposomes.

Since the partially proteolyzed pp60<sup>src</sup> did not bind to phospholipolipid liposomes it was possible to directly examine the role of phospholipolipid in the phosphorylation of AIIt without the potential complication of the binding of both pp60<sup>src</sup> and AIIt to the phospholipolipid liposomes. We therefore examined the specificity of the phospholipolipid stimulation of phosphorylation of AIIt by partially proteolyzed pp60<sup>src</sup>. As shown in Table II, phosphatidylerine and phosphatidylinositol liposomes stimulate the phosphorylation of AIIt by partially proteolyzed pp60<sup>src</sup>. In contrast, phosphatidylecine or phosphatidylenolamine liposomes do not stimulate the phosphorylation of AIIt. Therefore, this data provides direct evidence that phospholipolipid-induced conformational change in AIIt stimulates the tyrosine phosphorylation of AIIt by pp60<sup>src</sup>. Furthermore, since AIIt binds phosphatidylerine and phosphatidylinositol but not phosphatidylecine or phosphatidylenolamine (23), these results suggest that phospholipolipid binding and not a non-specific effect of phospholipolipid is responsible for the phospholipolipid stimulation of phosphorylation of AIIt by pp60<sup>src</sup>.

**Dephosphorylation of AIIt by Plasma Membrane Phosphatases—** Our results suggest that the binding of AIIt to plasma membrane activates a conformational change in AIIt which enhances exposure of the tyrosine-phosphorylation site of the protein, resulting in the stimulation of the phosphorylation of the protein by pp60<sup>src</sup>. Previous work from our laboratory has shown that the tyrosine phosphorylation of AIIt induces a conformational change in AIIt which results in the inability of the protein to bind F-actin or heparin or to bridge biological membranes (16). Therefore, it was unclear from these studies if the tyrosine-phosphorylation site of plasma membrane-bound, tyrosine-phosphorylated AIIt, was accessible to plasma membrane-associated protein tyrosine phosphatases. Since the binding of AIIt to plasma membrane stimulated the phosphorylation of AIIt by pp60<sup>src</sup>, it was important to ascertain if the plasma membrane-bound AIIt was a substrate for plasma membrane-associated protein tyrosine phosphatases. As shown in Fig. 4, the incubation of pp60<sup>src</sup>-phosphorylated AIIt with plasma membrane resulted in the dephosphorylation of AIIt. The dephosphorylation was very rapid and was essen-
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Phosphorylation reactions were conducted at 30 °C in the presence of 25 mM HEPES (pH 7.5), 10 mM MgCl₂, 50 μM CaCl₂, 1.2 μM AIIt, 1.6 μg/ml partially proteolyzed pp60^c-src, and 800 μg/ml of pure phospholipid liposomes (Materials and Methods). The reaction was initiated by the addition of 25 μM ATP and terminated after 30 min.

| Phospholipid          | Stoichiometry | mol phosphate/mol AIIt |
|-----------------------|---------------|------------------------|
| Phosphatidylserine    | 1.33 ± 0.31   |                        |
| Phosphatidylethanolamine | 0.26 ± 0.03   |                        |
| None                  | 0.25 ± 0.01   |                        |

Although the structure of AIIt-F-actin complex is unknown, it is reasonable to suggest that this conformation of AIIt will be distinct from other AIIt conformations. Within the context of our studies, our data suggests that the Tyr-23 phosphorylation site of AIIt is not fully exposed or accessible to pp60^c-src when AIIt is in the Ca²⁺-induced conformation or in the F-actin-induced conformation. In contrast, when AIIt is in the phosphatidylserine-induced conformation, the tyrosine-phosphorylation site is accessible to pp60^c-src. The binding of Ca²⁺ and phosphatidylserine to AIIt, which induces the Ca²⁺- and phosphatidylserine-induced conformation of AIIt, results in only a modest increased exposure of the tyrosine-phosphorylation site compared to the phosphatidylserine-induced conformation of AIIt.

We have also examined the possibility that the stimulation of the pp60^c-src-dependent AIIt phosphorylation by phosphatidylserine or plasma membrane is not due to a phospholipid-induced conformational change in AIIt but is due to the binding and therefore the enhanced concentration of pp60^c-src and AIIt on the phosphatidylserine liposomes. However, as shown in Table II, the phospholipid-dependent stimulation of phosphorylation of AIIt is also observed in the presence of a partially proteolyzed pp60^c-src. Since this truncated enzyme does not bind to phospholipid liposomes (Fig. 3), the phospholipid-stimulated phosphorylation of AIIt must be due to a direct effect of phospholipid on AIIt. Furthermore, since the phosphorylation of AIIt is stimulated only by phospholipids that bind to AIIt (Table II), such as phosphatidylserine and phosphatidylinositol, our data suggest that a phospholipid-dependent conformational change in AIIt is responsible for stimulation of the phosphorylation of AIIt by pp60^c-src.

The phosphorylation of other substrates of pp60^c-src has also been shown to be accelerated upon plasma membrane binding. Vinculin binds phosphatidylserine, phosphatidylglycerol, and phosphatidic acid and the binding of these phospholipids to vinculin correlates with a increase in the phosphorylation of vinculin by pp60^c-src (35). The stimulation of vinculin phosphorylation was shown to be due to a phospholipid-induced conformational change in the protein and these investigators concluded that phospholipid binding resulted in the increased accessibility of a phosphorylation site of vinculin to pp60^c-src.

Within the cell, AIIt has been shown to be localized to the cytoplasmic surface of the plasma membrane in the submembranous cytoskeleton (34). Furthermore, AIIt has been shown to interact with endosomes and transfection of Madin-Darby.
canine kidney cells with a dominant negative mutant form of AIIT causes translocation of AIIT and early endosomes to the cytoplasm (36, 37). Similarly, pp60c-src has also been shown to be localized at the inner surface of the plasma membrane (38–40) and more recently this enzyme has been localized to the endosomes of fibroblasts (41). Therefore, AIIT and pp60c-src exist in the same intracellular compartment. The intracellular localization of AIIT has been suggested to be due to two properties of AIIT observed in vitro, namely phospholipid binding and F-actin binding. Although it is possible that the binding of AIIT to biological membranes could utilize plasma membrane-associated F-actin as the AIIT-binding site, our results (Fig. 2) suggest that AIIT bound to F-actin is not appreciably phosphorylated by pp60c-src. This result therefore suggests that AIIT bound to the phospholipid component of the plasma membrane is regulated by tyrosine phosphorylation.

Previous results from our laboratory (2, 16) have established that the tyrosine phosphorylation of AIIT produces a large conformational change in AIIT which results in the alteration or inhibition of many of the biological activities of AIIT. It was unclear from these previous studies if the phosphorylation site of the plasma membrane-associated tyrosine-phosphorylated AIIT was accessible to phosphotyrosine phosphatases. The results presented in Fig. 4 suggest that the plasma membrane-bound form of AIIT is readily dephosphorylated by plasma membrane-associated phosphotyrosine phosphatases. Therefore, considering the established association of pp60c-src with the plasma membrane, it appears that the key elements for the regulation of AIIT by phosphorylation and dephosphorylation are present at the plasma membrane.

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