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Rapid binding of synapsin I to F- and G-actin

A study using fluorescence resonance energy transfer

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Synapsin I is a nerve terminal phosphoprotein which interacts with synaptic vesicles and actin in a phosphorylation-dependent manner. By using fluorescence resonance energy transfer between purified components labeled with fluorescent probes, we now show that the binding of synapsin I to actin is a rapid phenomenon. Binding of synapsin I to actin can also be demonstrated when synaptic vesicles are present in the medium and appears to be modulated by ionic strength and synapsin I phosphorylation.

Synaptic vesicle; Nerve terminal; Protein phosphorylation

1. INTRODUCTION

Synapsin I is a neuronal phosphoprotein which is specifically associated with the cytoplasmic surface of small synaptic vesicles [1–3]. In in vitro experiments, synapsin I has been shown to bind to the sides of actin filaments and to bundle them in a phosphorylation-dependent manner [4,5]. In addition, synapsin I is able to influence actin dynamics by binding to actin monomers and causing them to polymerize into filaments. This effect is greatly decreased after phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) [6–9]. Because of its ability to interact with synaptic vesicles and actin in a phosphorylation-dependent manner, synapsin I has been hypothesized to play a role in the regulation of neurotransmitter release by reversibly cross-linking synaptic vesicles to the nerve terminal cytoskeleton (for recent reviews, see [10,11]).

In order to further characterize the interaction of synapsin I with actin, we have studied the binding of synapsin I to actin monomers (G-actin) and to preformed actin filaments (F-actin) by fluorescence resonance energy transfer (FRET), a technique which allows one to follow the kinetics of the interaction. These studies reveal that the binding of synapsin I to actin monomers and filaments is a rapid phenomenon, consistent with a dynamic role of synapsin I in remodelling the actin network during synaptic activity.

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Abbreviations: FRET, fluorescence resonance energy transfer; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase I

2. MATERIALS AND METHODS

2.1. *Protein purification and subcellular fractionation*

Synapsin I was purified from bovine brain as described by Schiebler et al [12] and modified by Bähler and Greengard [4] and stored at −80°C in 200 mM NaCl; 25 mM Tris-HCl, pH 8.0. Purified dephosphorylated synapsin I was phosphorylated by CaM kinase II as previously described [12]. The average phosphorylation stoichiometry was 2.15 mol phosphate/mol synapsin I.

Actin was prepared from an acetone powder of rabbit skeletal muscle in buffer A (0.2 mM $CaCl_2$; 0.2 mM ATP; 0.5 mM NaN_3; 0.5 mM β-mercaptoethanol; 2 mM Tris-HCl, pH 8.0) as described [13] and further purified by gel filtration on a column of Sephadex G-150 (Pharmacia, Sweden) [14].

Synaptic vesicles were purified from rat forebrain through the step of chromatography on controlled-pore glass beads and depleted of endogenous synapsin I as previously described [2,12].

2.2. *Labeling of proteins*

Dephosphosynapsin I, phosphorylated synapsin I, ovalbumin and horseradish peroxidase (1–1.4 mg/ml) were dialyzed for 36 h at 4°C against 100 mM NaCl; 10 mM Tris-HCl, pH 8.0. Tetramethylrhodamine iodoacetamide (Molecular Probes, Eugene, USA) was dissolved in 1.5% dimethylsulfoxide and added to the protein at a final concentration of 0.5 mg/ml. Incubation was carried out for 4 h in the dark at 4°C under a nitrogen stream. The reaction was quenched by the addition of β-mercaptoethanol (0.5 mM final concentration), and unbound rhodamine was removed by dialysis for 48 h at 4°C against 200 mM NaCl; 25 mM Tris-HCl, pH 8.0. The concentration of rhodaminated synapsin I was determined spectrophotometrically, and

was in the range 0.5–1.2 mg/ml, with a rhodamine/synapsin I molar ratio of approximately 0.3.

G-actin (1 mg/ml) was dialyzed for 36 h at 4°C against buffer A without β-mercaptoethanol and NaN_3, and subsequently mixed with 5-iodoacetamide-fluorescein (Molecular Probes, Eugene, USA) previously dissolved in 1 5% acetone (1 mg/ml final concentration). The incubation was carried out for 12 h at 4°C under nitrogen in the dark. β-mercaptoethanol (0.5 mM final concentration) was then added, and the preparation was dialyzed for 36 h against buffer A. KCl and $MgCl_2$ (final concentrations 100 and 1 mM, respectively) were added and actin was allowed to polymerize for 30 min at room temperature. Unbound fluorescein and unpolymerized actin were removed by centrifuging the sample for 20 min at 75,000 rpm in a Beckman TL100.3 rotor, followed by resuspension of the pellet in buffer A and dialysis for 36 h at 4°C against the same buffer. The concentration of fluoresceinated actin was calculated to be in the range of 4–5 mg/ml, with a fluorescein/actin ratio close to unity.

2.3. *Fluorescence measurements*

The binding of rhodaminated synapsin I to fluoresceinated actin was evaluated by FRET between the two fluorochromes The formation of a synapsin I-actin complex was followed by observing the quenching in the fluorescein emission peak induced by rhodamine. Fluorescence measurements were performed in a LS-50 spectrofluorometer (Perkin-Elmer, UK). The samples in 1.5 ml quartz cuvettes were excited at a wavelength ranging from 410 to 450 nm and emission spectra were recorded. Excitation and emission slit widths were set at 5 and 10 nm, respectively. When the kinetics of the interaction was to be measured, readings were taken every 0.1 s, and the emission was monitored at 520 nm, which corresponds to the peak of emission of fluoresceinated actin. All measurements were performed under continuous stirring.

2.4. *Other methods*

Protein concentrations were determined by the method of Lowry et al. [15], the method of Bradford [16] or spectrophotometrically using an $E^{1\%}_{290}$ pf 6.5 for actin [17] and an $E^{1\%}_{277}$ of 6.7 for synapsin I [18]. In order to calculate the kinetic parameters, binding curves were fitted using the computer program Quattro Pro (Borland, USA).

3. RESULTS

Binding of synapsin I to actin was measured by monitoring the decrease in fluorescence emission of fluoresceinated actin upon incubation with rhodaminated synapsin I. Fig. 1 shows the results obtained by incubating fluoresceinated F-actin with rhodaminated synapsin I under either high (100 mM KCl) or low (30 mM KCl) ionic strength conditions. Upon excitation at 410 nm, a consistent decrease in the emission peak of fluorescence was observed under both conditions (upper panel, curves a and d, and lower panel, curves a and b, respectively). The quenching was specific for synapsin I, since neither rhodaminated ovalbumin (Fig.1, upper panel, curve b) nor rhodaminated horseradish peroxidase (not shown) induced a comparable degree of quenching.

The interaction of synapsin I with actin filaments is known to induce actin bundle formation. It therefore seemed important to test whether the observed quenching of actin fluorescence was due to the presence of bundles. For this purpose, we incubated fluoresceinated actin with rhodaminated ovalbumin in the presence of unlabeled dephosphosynapsin I. Under these conditions, only a 10% decrease in fluorescence emission was observed (Fig.1, upper panel, curve c), although the presence of large actin bundles was clearly visible in the sample.

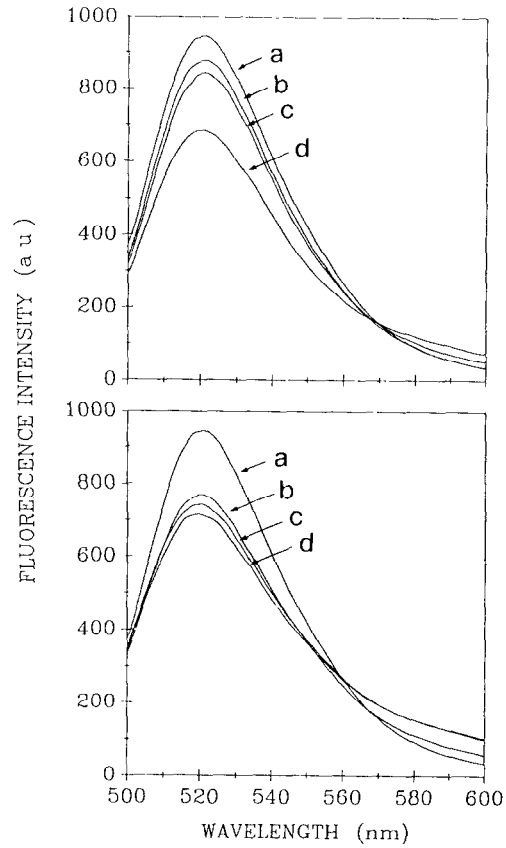

Fig 1. Quenching of the fluorescence emission spectrum of fluoresceinated actin by rhodaminated synapsin I. Upper panel: The specificity of FRET between fluorescein-labeled F-actin and rhodamine-labeled synapsin I was tested by measuring the emission spectrum of fluoresceinated F-actin (2.5 μM, polymerized by an overnight incubation at 4° C in buffer A supplemented with KCl and $MgCl_2$) incubated in the presence of various proteins. The final composition of the medium was (in mM): KCl, 100; $MgCl_2$, 1; ATP, 0.2; $CaCl_2$, 0.03, NaCl, 6; Tris-HCl, 2.75, pH 7.4. The excitation wavelength was 410 nm. (a, actin alone; b, actin plus 300 nM rhodaminated ovalbumin; c, actin plus 300 nM rhodaminated ovalbumin and 300 nM unlabeled synapsin I; d, actin plus 300 nM rhodaminated synapsin I). Lower panel: FRET between fluorescein-labeled F-actin (2.5 μM) and rhodamine-labeled synapsin I (164 nM) in the presence of 0.2 mg/ml synaptic vesicles depleted of endogenous synapsin I. The final composition of the medium was (in mM): KCl, 30; $MgCl_2$, 1, ATP, 0.2; $CaCl_2$, 0 03; NaCl, 6; Tris-HCl, 2 75; glycine, 24; Hepes NaOH,0 4, pH 7 4. (a, actin alone; b, actin plus rhodaminated synapsin I; c and d, actin plus rhodaminated synapsin I and unlabeled synaptic vesicles. In c, actin was preincubated for 15 min on ice with rhodaminated synapsin I prior to the addition of unlabeled synaptic vesicles; in d, rhodaminated synapsin I was preincubated for 15 min on ice with unlabeled synaptic vesicles prior to being added to the actin solution).

The presence of unlabeled synaptic vesicles in the medium did not modify the ability of synapsin I to interact with actin, independently of whether they had been preincubated with synapsin I prior to adding actin

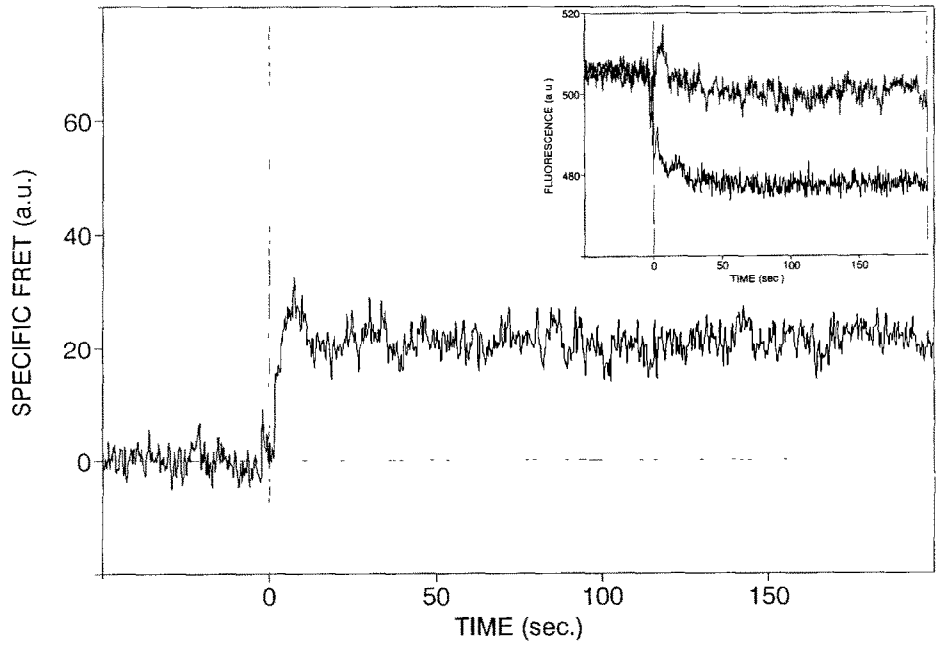

Fig. 2. Time course of the binding of synapsin I to F-actin. Fluoresceinated actin was polymerized overnight at 4°C and subsequently equilibrated for 1 h at 8°C before adding either rhodaminated or unlabeled dephosphosynapsin I. The time course of fluorescence quenching at 8°C was followed by continuous monitoring (with sampling at 0.1 s intervals) of the emission at 520 nm (excitation wavelength: 420 nm). Final concentrations were: fluoresceinated actin, 750 nM; dephosphosynapsin I, 75 nM; KCl, 100 mM; $MgCl_2$, 1 mM; NaCl, 6 mM; ATP, 0.2 mM; $CaCl_2$, 0.01 mM; Tris-HCl, 2.75 mM, pH 7.4. Results are expressed as specific FRET, resulting from the subtraction of the curve obtained with unlabeled synapsin I from the curve obtained with rhodaminated synapsin I (upper and lower curve in the inset, respectively)

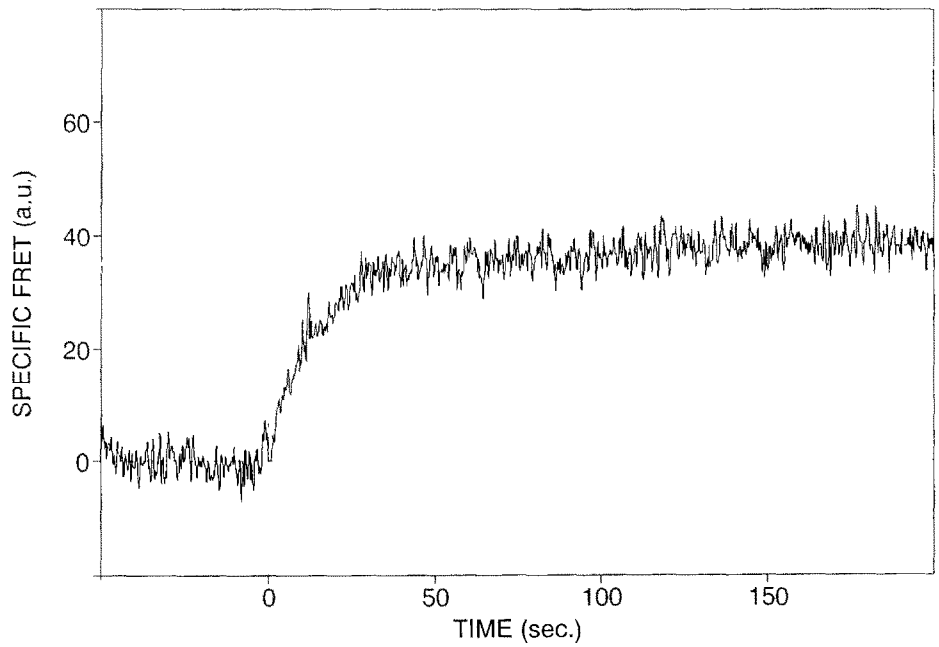

Fig. 3. Time course of the binding of synapsin I to G-actin. The quenching of fluorescence emission of 750 nM fluoresceinated G-actin induced by the addition of 75 nM rhodaminated synapsin I was measured in the absence of K^+ and Mg^{2+}. Experimental conditions were as described in the legend to Fig. 2, except that KCl and $MgCl_2$ were replaced by 76 mM NaCl. The results are expressed as specific FRET, resulting from the subtraction of the curve obtained with unlabeled synapsin I from the curve obtained with rhodaminated synapsin I.

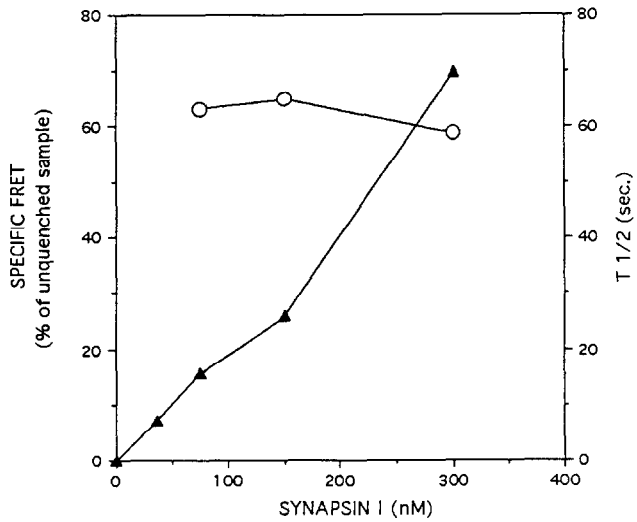

Fig. 4. Effect of increasing concentrations of synapsin I on the binding to G-actin. The quenching of fluorescence emission of 750 nM fluoresceinated G-actin induced by the addition of various concentrations of rhodaminated synapsin I was measured in a buffer containing. 0.2 mM ATP, 6 mM NaCl, 0.2 mM $CaCl_2$ and 2.75 mM Tris-HCl, pH 7.4. ▲, specific FRET expressed in percentage of the fluorescence intensities measured at steady state in the samples containing unlabeled synapsin I; ○, $T_{\frac{1}{2}}$ (in seconds).

or whether they were added to a synapsin I-actin mixture (Fig. 1, lower panel).

The association reaction between synapsin I and actin filaments proceeded very rapidly. At 37°C, the reaction was too fast for its kinetics to be evaluated. Therefore, the reaction was slowed down by decreasing the temperature to 8°C. Under this condition, the half-time of the association reaction was found to be of the order of 2 seconds (Fig. 2).

The kinetics of association of synapsin I with actin monomers was measured by adding rhodaminated synapsin I to fluoresceinated G-actin in the absence of KCl and $MgCl_2$, conditions under which actin polymerization is highly unfavourable [6,7]. When the experiment was carried out under high ionic strength conditions (76 mM NaCl), a rapid quenching of the fluorescence emission of actin was observed, the half-time of the association reaction being 9 seconds (Fig. 3). At low ionic strength (6 mM NaCl), the final extent of quenching was higher, but the reaction appeared somewhat slower (Fig. 4).

The degree of quenching was dependent on the concentration of synapsin I both in the case of the binding to actin monomers and in the case of the binding to actin filaments (Figs. 4 and 5). However, in the ranges of synapsin I:actin stoichiometries tested, the concentration of synapsin I did not significantly influence the rapidity of association (Fig.4 and not shown).

Under all concentrations tested, the extent of fluoresceinated actin quenching was higher for dephosphosynapsin I than for synapsin I phosphorylated by CaM kinase II (Fig. 5), consistent with the previously reported decrease in the number of synapsin I binding sites on actin filaments induced by phosphorylation [4].

4. DISCUSSION

In the present paper FRET was utilized to evaluate the interaction of synapsin I with actin filaments and monomers. FRET is a powerful and widely used technique for the evaluation of the dynamics of molecular assembly, which offers the advantages of being non-perturbing, highly specific and allowing a kinetic evaluation of protein-protein interactions [19]. The data indicate that synapsin I associates very rapidly with both preformed actin filaments and actin monomers. At 37°C, synapsin I binding to actin was too rapid to be measured with the available techniques; therefore we had to operate at reduced temperatures. The rapidity of association was maximal at physiological ionic strength, although the extent of synapsin I association was higher at low ionic strength. In addition, the association of synapsin I with actin filaments was modulated by synapsin I phosphorylation.

The presence of unlabeled synaptic vesicles depleted of endogenous synapsin I in the medium did not modify the FRET between synapsin I and actin, suggesting that synapsin I is able to interact with actin also when it is in the membrane-bound form. These findings are consistent with a physiological role of synapsin I in binding

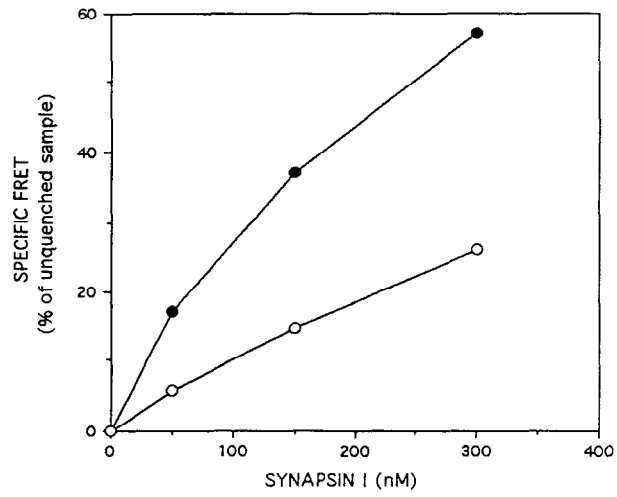

Fig 5. Effect of synapsin I phosphorylation on FRET. Fluoresceinated actin (750 nM) was polymerized overnight at 4°C and subsequently equilibrated for 1 h at 8°C before adding the indicated concentrations of rhodaminated dephosphosynapsin I (●) or phosphosynapsin I (○). Experimental conditions were as described in the legend to Fig. 2 (excitation wavelength: 450 nm). Results are expressed as percentage of fluorescence quenching at steady state after subtraction of the values obtained with unlabeled dephospho- or phospho-synapsin I.

synaptic vesicles to the actin-based cytoskeletal network of the nerve terminal.

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