Identification of a dynein molecular motor component in *Torpedo* electroplax; binding and phosphorylation of Tctex-1 by Fyn

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Abstract The microtubule protein Tctex-1 was cloned from *Torpedo* electroplax, a biochemical model of the neuromuscular junction, using the unique domain of Fyn in the yeast two hybrid system. Binding of Tctex-1 and Fyn also occurred in vitro. *Torpedo* Tctex-1 was contained within the molecular motor protein dynein. A Src class kinase was also complexed with dynein. Tctex-1 was enriched in electric organ vs. skeletal muscle, was present in the postsynaptic membrane, and coprecipitated with the acetylcholine receptor. The sequence of Tctex-1 contained a tyrosine phosphorylation motif and Tctex-1 could be phosphorylated by Fyn in vitro and in vivo. These data demonstrated that Tctex-1-containing dynein is a cytoskeletal element at the acetylcholine receptor-enriched postsynaptic membrane and suggested that Tctex-1 may be a substrate for Fyn.

Key words: Neuromuscular junction; Synaptogenesis; Microtubule; Phosphorylation; Protein tyrosine kinase

1. Introduction

Protein phosphorylation is recognized as a primary post-translational mechanism for the regulation of essentially all cellular processes [1,2]. Protein tyrosine kinases are highly expressed in the nervous system suggesting they are involved in the regulation of synaptic transmission [3,4]. The neuromuscular junction (NMJ) has served as a classic model system for studying synapse formation and function. The acetylcholine receptor (AChR) is the ligand gated ion channel that mediates rapid depolarization in the postsynaptic skeletal muscle cell membrane. Recent studies have indicated important roles for protein tyrosine kinases in regulating the expression, localization, and channel kinetics of the AChR [5–7]. We have previously identified two protein tyrosine kinases of the Src family, Fyn and Fyk, that are highly expressed at the endplate. Fyn and Fyk bind to and phosphorylate the AChR, thereby supporting the importance of Src class kinases at the NMJ [8,9].

In this study, using *Torpedo californica* electric organ, a biochemical model for the neuromuscular junction, we sought to identify postsynaptic substrates and/or regulators of Fyn and Fyk by characterizing components that interact directly with the kinases. Src family kinases each contain a unique region, a Src homology two (SH2) domain, a Src homology three (SH3) domain, and a catalytic domain. We have searched for postsynaptic components that interact with specific subdomains of Fyn and Fyk by screening a *Torpedo* electric organ library using the yeast two hybrid cloning system. With this strategy, we have identified a microtubule molecular motor component of dynein, *Torpedo* Tctex-1, that interacts with the unique domain of Fyn and may be a substrate for this protein tyrosine kinase.

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Abbreviations: NMJ, neuromuscular junction; AChR, acetylcholine receptor; SH2, Src homology 2; SH3, Src homology 3; GST, glutathione *S*-transferase; pBK-CMV, pBK-CMVΔlac; CT-SrcK, carboxy terminal pan Src kinase antibody

The nucleotide sequence for *Torpedo* Tctex-1 has been deposited in the GenBank data base under GenBank Accession Number AF086756.

2. Materials and methods

2.1. Preparation of Torpedo electric organ pPC86 library

A *Torpedo californica* electric organ plasmid library was constructed using the yeast expression vector pPC86 which contains the GAL4 transcriptional activating domain [10]. Electric organ poly(A)$^+$ RNA was reverse transcribed and the cDNA directionally cloned into pPC86 using the SuperScript System (Gibco). The plasmid library was transformed into DH10B cells resulting in the generation of 1.4×10^6 independent clones.

2.2. Yeast two-hybrid system screening

The subdomains of Fyn and Fyk including the unique domain of Fyn were amplified by PCR using pBS-Fyn or pBS-Fyk [8] as templates and subcloned into the *Sal*I and *Not*I sites of the pPC97 vector which contains the GAL4 DNA binding domain. The plasmids were cotransformed with the *Torpedo* electric organ pPC86 library into the PCY2 yeast strain [10] and positive clones were selected on leu$^-$trp$^-$ plates and assayed for β-galactosidase activity. Positive clones were analyzed by dideoxysequencing and identity with known nucleotide and amino acid sequences was determined using BLAST from NCBI. Protein characteristics were determined with Prosite.

2.3. Generation of anti-Tctex-1 antibodies

A glutathione *S*-transferase (GST) fusion protein containing the complete protein sequence of Tctex-1 was prepared and used to produce antiserum generated in rabbits as described [8]. Crude serum was affinity purified using Tctex-GST fusion protein resolved by SDS-PAGE and immobilized onto PVDF by transblotting as described [11].

2.4. Tissue preparations

To examine the distribution of Tctex-1, *Torpedo* tissue homogenates, membranes, and cytosolic fractions were prepared as previously described [12].

2.5. QT-6 cell transfection

QT-6 cells, kindly provided by J.H. Steinbach (Washington University), were cultured as described [13]. Tctex-1 and Fyn cDNAs were subcloned into the pBK-CMVΔlac (pBK-CMV) vector engineered as described [14]. The Tctex-1, Fyn, or empty pBK-CMV vectors were transfected into QT-6 cells using the calcium phosphate coprecipitation method [15].

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2.6. Immunoprecipitations

Aliquots of *Torpedo* tissues or transfected QT-6 cells were solubilized with buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4 (Lysis Buffer). In some experiments, total cellular Tctex-1 was analyzed by solubilizing transfected cell proteins with Lysis Buffer without Triton X-100 but containing 1% SDS followed by a 1:7.5 dilution in Lysis Buffer and sonication for 20 s. The lysates were centrifuged at $100\,000\times g$ for 10 min and the supernatants immunoprecipitated with anti-Tctex-1 serum as previously described [9].

2.7. Fusion protein affinity chromatography

Fyn-GST, Fyk-GST, and Tctex-GST fusion proteins and GST backbone protein were prepared and used as affinity reagents as previously described [9]. Bound proteins were either eluted with SDS-PAGE sample buffer and analyzed by Western blotting or used for phosphorylation assay as described below.

2.8. Western blotting

Western blots were performed with primary antibody at 1:100 for affinity purified anti-Tctex-1 antibody, 1:5000 for anti-phosphotyrosine antibody (UBI), 1:2000 for the monoclonal 74.1 anti-dynein antibody [16], 1:1000 for the monoclonal 1234 anti-rapsyn antibody [17], 1:10 000 for the monoclonal SYN1351 anti-syntrophin antibody [18], 1:400 for the carboxy terminal pan Src kinase antibody SRC2 (CT-SrcK) from Santa Cruz Biotechnology followed by the appropriate secondary antibody and ECL using BioMax MR-1 film. Densitometry scanning was performed on a Howtek Scanner using PDI Discovery Series software at the Lombardi Cancer Center's Macromolecular Synthesis and Sequencing Shared Resource which is supported in part by US Public Health Service Grant P30-CA-51008.

2.9. Protein tyrosine kinase assays

The binding of a protein tyrosine kinase activity to Tctex-1 GST fusion protein was examined using the synthetic substrate poly(Glu-Na,Tyr), 4:1 (Sigma), and [γ-^{32}P]ATP as previously described [8].

To examine phosphorylation of Tctex-1 fusion protein, *Torpedo* electric organ postsynaptic membrane proteins were solubilized and immunoprecipitated with anti-Fyn, anti-Fyk, or preimmune serum as described above. After washing, the immunoprecipitates were incubated with Tctex-GST fusion or GST backbone protein (2 μg each) with [γ-^{32}P]ATP under phosphorylating conditions as described [8]. The supernatants were analyzed by 10% SDS-PAGE and autoradiography using BioMax MR-1 film. Phosphoamino acid analysis was performed as described [8].

2.10. Protein analysis

Cellular protein levels were determined by the method of Lowry [19] while fusion protein concentrations were determined as described [9].

3. Results

3.1. Cloning of a Torpedo electric organ component using the unique domain of Fyn in the yeast two hybrid screening system

Our major goal was to identify proteins that are involved in the action of Fyn and Fyk at the NMJ of skeletal muscle by molecularly cloning *Torpedo* electric organ components that interact directly with these protein tyrosine kinases. The subdomains of Fyn and Fyk in the pPC97 vector were used to screen a *Torpedo* electric organ pPC86 library as described in Section 2. Upon screening with the unique domain of Fyn containing amino acid residues 9–92 [8], 38 colonies out of 4×10^5 plasmids screened survived the selection process of which 20 were positive for β-galactosidase activity. Twelve colonies were cloned to homogeneity. Four of the clones had the identical nucleotide sequence in the coding region as presented in Fig. 1A. However, within the non-coding region, two of the four clones had the identical subcloning sites while the other two each had unique sites

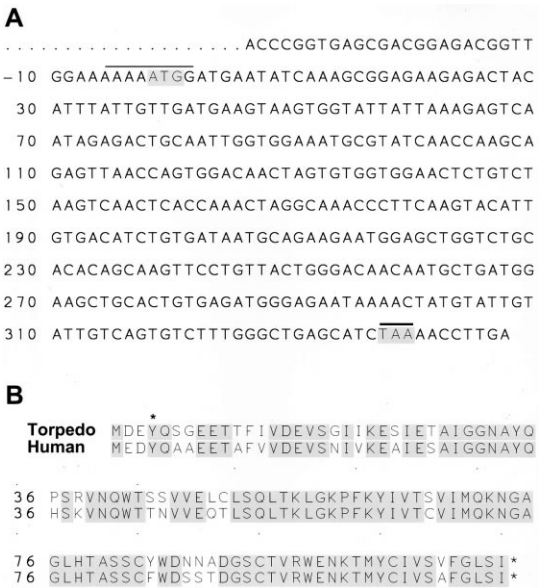

Fig. 1. Nucleotide and amino acid sequence of *Torpedo* Tctex-1. A cDNA clone obtained by screening a *Torpedo* electric organ library with the unique domain of Fyn using the yeast two hybrid system as described in Section 2 was analyzed by dideoxysequencing. A: The nucleotide sequence; the putative start codon is indicated by shading while the stop codon is indicated by shading with a heavy bar, and the Kozak motif is indicated by a fine bar. B: The homology of the deduced amino acid sequence of the clone identified in A with known sequences was determined as described in Section 2. The amino acid identities between *Torpedo* Tctex-1 and human Tctex-1 [20] are indicated by shading. Y*, putative phosphorylation site.

demonstrating the identification of three individual pPC86 constructs.

The deduced amino acid sequence indicated the cDNA to encode a protein of 12.4 kDa and a p*I* of 4.6. No signal sequence or N-terminal acylation sites were detected; the polypeptide sequence suggested a cytosolic protein. Homology searches demonstrated that the nucleotide (data not shown) and deduced amino acid sequences were most similar to human Tctex-1 ([20]; Fig. 1B). The coding region at the nucleotide and protein level showed 70% and 80% identity, respectively, with human Tctex-1. In addition, there was 92% conservation at the protein level with human Tctex-1. Thus, *Torpedo* Tctex-1 with 70% identity was slightly more homologous to human Tctex-1 than is *Chlamydomonas* Tctex-1 which has 62% identity with human Tctex-1 [21]. Tctex-1, originally identified as a gene of the mouse *t*-complex sterility locus [22] is a component of the microtubule-associated molecular motor protein dynein [11,21]. These data indicated that the cDNA clone identified with the unique domain of Fyn encoded the *Torpedo* form of Tctex-1.

3.2. Tissue and subcellular distribution of Torpedo Tctex-1

If *Torpedo* Tctex-1 is a substrate or regulator of Fyn, the protein should be expressed in the appropriate tissues and subcellular compartments. To examine the expression of Tctex-1 at the protein level, we generated antibodies to a bacterial Tctex-GST fusion protein as described in Section 2. These antibodies recognized a 13-kDa protein of *Torpedo* electric organ as demonstrated by Western analysis. The binding of the antibodies during Western blotting was blocked by

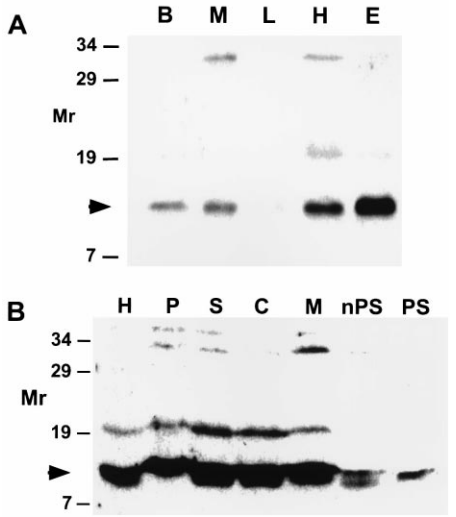

Fig. 2. Tissue and subcellular distribution of Tctex-1. A: Total homogenates of the indicated *Torpedo* tissues were prepared as described in Section 2. Aliquots of each tissue homogenate representing 20 μg of protein were resolved by 15% SDS-PAGE and analyzed by Western blotting using anti-Tctex-1 antibody as described in Section 2. Tissues analyzed were brain (B), muscle (M), liver (L), heart (H), and electric organ (E). Arrow indicates the position of Tctex-1. B: *Torpedo* electric organ tissue was fractionated as described in Section 2. Aliquots of the indicated fractions representing 25 μg (H), or 75 μg (P, S, C, M, nPS, PS) were resolved by 15% SDS-PAGE and analyzed by Western blotting using anti-Tctex-1 antibody as described in Section 2. Fractions analyzed were total homogenate (H), low speed pellet (P), low speed supernatant (S), cytosol (C), total membranes (M), non-postsynaptic membranes (nPS), and postsynaptic membranes (PS). Arrow indicates the position of Tctex-1.

fusion protein used to generate the anti-serum but not by backbone GST protein indicating that the 13-kDa band was in fact Tctex-1 (data not shown). In some experiments, the antibodies also detected higher molecular weight aggregates of Tctex-1 (see Fig. 2A,B). Tctex-1 was detected in *Torpedo* electric organ, heart, skeletal muscle, and brain but not liver (Fig. 2A). This pattern of tissue specific expression was identical to the expression of Fyn at the protein and/or RNA level as previously demonstrated [8]. The level of Tctex-1 was highest in electric organ, which is enriched in postsynaptic components including the AChR. In addition, Tctex-1's high expression in electric organ and low expression in muscle agree with the ratio of AChR and Fyn expression in these two tissues. These data demonstrated that the tissue distribution of Tctex-1 agreed with the distribution of Fyn. In addition, the ratio of Tctex-1 in electric organ vs. muscle as well as the high level of Tctex-1 in electric organ suggested that this cytoskeletal protein may have a function at the postsynaptic membrane.

The deduced sequence of Tctex-1 indicated a soluble cytoplasmic protein. However, Fyn is membrane bound [8]. To determine the cellular localization of Tctex-1, subcellular fractions of *Torpedo* electric organ were analyzed by Western blotting using anti-Tctex-1 antibody. Tctex-1 was ubiquitously expressed within the electroplax being detected in the low speed pellet and supernatant, the cytosolic fraction, and the total membrane fraction (Fig. 2B). Tctex-1 was distributed equally between the cytosolic and total membrane fraction demonstrating Tctex-1 to be both soluble and membrane associated (Fig. 2B). *Torpedo* electroplax membranes can be fractionated on discontinuous sucrose gradients to enrich for postsynaptic vs. non-postsynaptic membranes [23,24]. Tctex-1 was found in both the postsynaptic and non-postsynaptic membrane fractions (Fig. 2B). The fact that more Tctex-1 was detected in the total membrane pellet than the postsynaptic plus non-postsynaptic membrane fractions suggested that Tctex-1 may have dissociated from the membranes during the prolonged sucrose gradient purification. This observation was consistent with Tctex-1 being membrane associated primarily via protein-protein interaction rather than by a posttranslational anchoring to the membrane. Fyn is found not in the cytosol but in the postsynaptic and non-postsynaptic membranes [8]. Although Tctex-1 was found in the cytosol, it was also colocalized with Fyn in both the postsynaptic and non-postsynaptic membranes of electric organ. Thus, the subcellular distribution of Tctex-1 suggested cytoplasmic and membrane functions including a function at the postsynaptic membrane.

3.3. *Expression of a Tctex/dynein complex in Torpedo electric organ*

Mouse Tctex-1 is a light chain of the brain cytoplasmic microtubule molecular motor protein dynein [11] as well as flagellar dynein [21]. Cytoplasmic dynein is believed to be required for retrograde transport, away from the membrane, in both neuronal and non-neuronal cells (for reviews see [25,26]). We tested whether *Torpedo* Tctex-1 was complexed with dynein or cytoskeletal elements of the NMJ including rapsyn and syntrophin. Precipitation of Tctex-1 from electric organ homogenate resulted in coprecipitation of the intermediate chain, IC74, of dynein (Fig. 3A). Scanning densitometry analysis demonstrated that under these conditions, $56 \pm 15\%$ (mean ± S.E.M.; n=3) of the dynein IC74 in electric organ was complexed with Tctex-1. The coprecipitation of Tctex-1 and dynein was specific since dynein was not precipitated by preimmune serum and neither rapsyn nor syntrophin coprecipitated with Tctex-1 (Fig. 3A). Further evidence for Tctex-1 being a component of dynein in *Torpedo* electric organ was obtained by immunoprecipitating dynein IC74 and analyzing the precipitate for Tctex-1 by Western blotting (Fig. 3B). Tctex-1 was specifically coprecipitated with dynein. Densitometry scanning analysis showed that $66 \pm 4\%$ (mean ± range; $n = 2$) of Tctex-1 was contained within the dynein complex. These data indicated that Tctex-1 was a component of the molecular motor protein dynein in *Torpedo* electric organ.

Association of Src class kinases with the Tctex/dynein complex was also tested. The presently available anti-Fyn antibodies were not useful for directly demonstrating association of Fyn with the Tctex/dynein complex. However, in four experiments, precipitation of dynein from electric organ homogenate resulted in coprecipitation of a Src family kinase as demonstrated by Western analysis with an antibody to the carboxy terminal sequence of Src family kinases (CT-Src antibody) (Fig. 3C). Coimmunoprecipitation of the Src class kinase with dynein was specific since no kinase was precipitated with empty protein G-Sepharose beads. Densitometry scanning demonstrated that $2.5 \pm 0.5\%$ (mean ± range; $n = 2$) of the electric organ kinase(s) recognized by the CT-Src antibody was coprecipitated with the dynein complex. Library screening as well as PCR analysis has identified Fyn and Fyk as the only Src class kinases in *Torpedo* electric organ [8]. Thus,

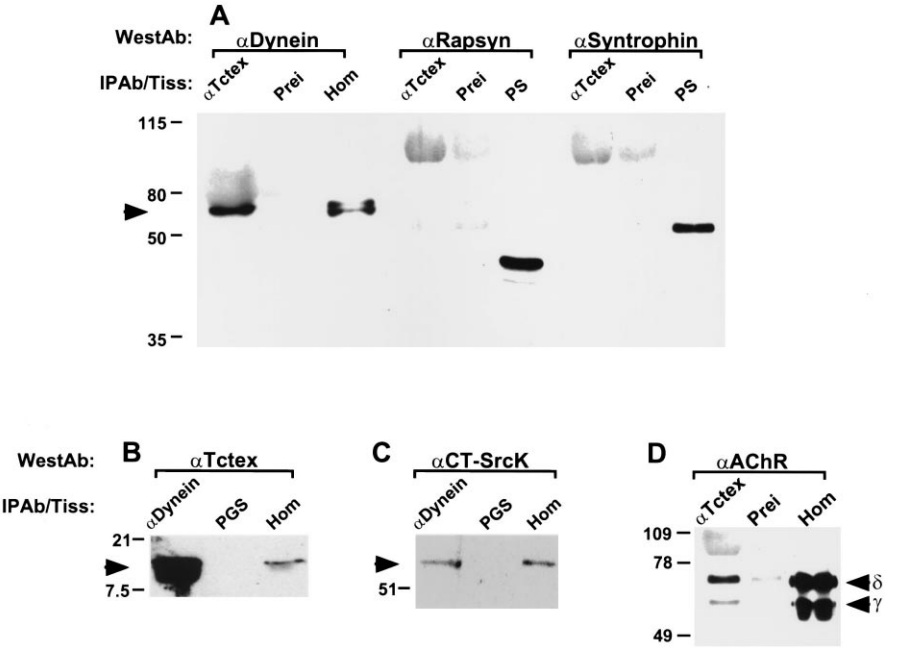

Fig. 3. Characterization of a Tctex-1/Dynein complex in *Torpedo* electric organ. A: Aliquots of *Torpedo* electric organ homogenate representing 300 μg of protein were solubilized and immunoprecipitated with anti-Tctex-1 (αTctex) or preimmune (Prei) serum as described in Section 2. The precipitates and 30 μg of homogenate (Hom) or 0.3 μg of postsynaptic membranes (PS) were resolved by 10% SDS-PAGE and analyzed by Western blotting using an anti-dynein IC74 (αDynein), anti-rapsyn (αRapsyn), or anti-syntrophin (αSyntrophin) antibody. Arrow indicates the position of dynein IC74. B: *Torpedo* electric organ homogenate representing 500 μg of protein was solubilized and immunoprecipitated with anti-dynein IC74 (αDynein) antibody or empty protein G-Sepharose (PGS). The precipitates and 30 μg of solubilized homogenate (Hom) were resolved by 15% SDS-PAGE and analyzed by Western blotting using an anti-Tctex-1 antibody (αTctex) as described in Section 2. Arrow indicates the position of Tctex-1. C: *Torpedo* electric organ homogenate representing 2 mg of protein was solubilized and immunoprecipitated with anti-dynein IC74 (αDynein) antibody or empty protein G-Sepharose (PGS). The precipitates and 60 μg of solubilized homogenate (Hom) were resolved by 8% SDS-PAGE and analyzed by Western blotting using an anti-CT-Src kinase antibody (αCT-SrcK) as described in Section 2. Arrow indicates the position of Src kinases. D: *Torpedo* electric organ homogenate representing 300 μg of protein was solubilized and immunoprecipitated with anti-Tctex-1 (αTctex) or preimmune (Prei) serum. The precipitates and 3.0 μg of solubilized homogenate (Hom) were resolved by 10% SDS-PAGE and analyzed by Western blotting using a monoclonal anti-AChR antibody (88 b) as described in Section 2. Arrows indicate the position of AChR δ and γ subunits. In each panel, molecular weights are indicated to the left.

these data suggested that the Src class kinase associated with the Tctex/dynein complex was Fyn and/or Fyk.

The primary function of the endplate at the NMJ is to mediate synaptic transmission via the activation of the AChR. Since Tctex-1 was detected in postsynaptic membranes, complex formation between the cytoskeletal protein and the AChR was examined. Immunoprecipitation of Tctex-1 resulted in coprecipitation of the AChR (Fig. 3D). Coprecipitation of the AChR with Tctex-1 was specific since little AChR was precipitated by preimmune serum (Fig. 3D). In addition, the cytoskeletal protein rapsyn, which is stoichiometrically expressed with the AChR, was not coprecipitated with Tctex-1 (Fig. 3A). Scanning densitometry analysis demonstrated that $0.12 \pm 0.04\%$ (mean ± S.E.M.; n=3) of the AChR was specifically coimmunoprecipitated with Tctex-1. These data indicated that the AChR was also contained in a complex with Tctex-1.

3.4. Binding of Tctex-1 to Fyn

Results from the yeast two hybrid screen suggested that Tctex-1 bound to Fyn via the unique domain of the kinase. To confirm these results, the ability of Tctex-1 and Fyn to bind in vitro was tested. Initially, we determined whether a protein tyrosine kinase activity could bind to Tctex-GST fusion protein using the synthetic polypeptide substrate poly-(Glu-Na,Tyr), 4:1, as described in Section 2. Solubilized *Torpedo* electric organ postsynaptic membrane proteins containing Fyn, were incubated with Tctex-GST fusion or GST backbone protein affinity resins and the bound proteins analyzed for protein tyrosine kinase activity using [γ-^{32}P]ATP. Specific protein tyrosine kinase activity was taken as the difference in filter-bound radioactivity in the presence or absence of substrate as described in Section 2. In three experiments, phosphorylation of poly(Glu-Na,Tyr), 4:1, occurred only upon incubation of the substrate with proteins bound to Tctex-GST fusion protein affinity resin; the synthetic substrate was not phosphorylated by proteins bound non-specifically to GST backbone protein affinity resin (Fig. 4A). These data demonstrated that a protein tyrosine kinase activity bound to Tctex-1 in vitro.

The ability of Tctex-1 to bind Fyn in vitro was further tested using Fyn fusion protein affinity chromatography. A Fyn unique domain-GST fusion protein bound to glutathione agarose was prepared and incubated with *Torpedo* electric organ cytosolic proteins containing Tctex-1. Tctex-1 bound to the Fyn unique domain affinity resin as demonstrated by Western blotting (Fig. 4B). Scanning densitometry analysis demonstrated that, under these conditions, 5% of the solubilized Tctex-1 bound to the Fyn fusion protein. The binding was specific for the Fyn unique domain since Tctex-1 did not bind to either the backbone GST protein or the Fyk unique domain-GST fusion protein (Fig. 4B). These in vitro data

support binding of Tctex-1 and Fyn as indicated by interaction of Fyn and Tctex-1 using the two hybrid approach.

3.5. Phosphorylation of Tctex-1 by Fyn

The biological importance of an interaction between Tctex-1 and Fyn could be that Tctex-1 may be a substrate for Fyn or that Fyn kinase activity may be regulated by Tctex-1. The deduced amino acid sequence of Tctex-1 revealed a consensus site for tyrosine phosphorylation at Tyr[4] (Fig. 1). The putative Tctex-1 phosphorylation site was similar to the phosphorylation site of the AChR β-subunit of *Torpedo* which is a direct substrate for Fyn in vitro [27,8]. To test whether Tctex-1 could be phosphorylated by Fyn in vivo, we examined the effect of coexpressing Tctex-1 with Fyn in a heterologous system. When QT-6 fibroblasts were transfected with Tctex-1 plus Fyn, Tctex-1 was phosphorylated on tyrosine residue(s) as demonstrated by anti-phosphotyrosine Western blotting (Fig. 5A). The phosphorylation was dependent on Fyn since Tctex-1 immunoprecipitated from cells not cotransfected with the kinase was not phosphorylated.

To determine whether the ability of Fyn to mediate Tctex-1 phosphorylation was due to a direct phosphorylation of the microtubule protein by the kinase, the ability of Fyn to phosphorylate a Tctex-1 fusion protein in vitro was tested. Fyn and Fyk, immunoprecipitated from *Torpedo* electric organ

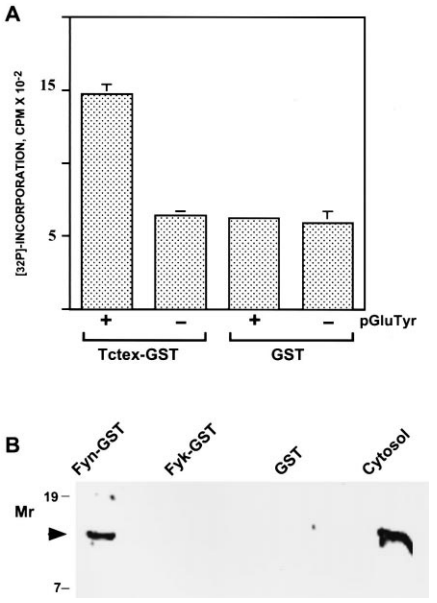

Fig. 4. In vitro binding of Tctex-1 and Fyn. A: *Torpedo* electric organ postsynaptic membranes representing 200 μg of membrane protein, were solubilized and incubated with glutathione-agarose containing 2 μg of either Tctex-1-GST fusion (Tctex-GST) or GST backbone (GST) protein as described in Section 2. After washing, the pellets were incubated with (+) or without (−) poly(Glu-Na, Tyr), 4:1, at 1 mg/ml under phosphorylating conditions in the presence of [γ-^{32}P]ATP. After 30 min at 30°C, the reactions were stopped, and the supernatants were analyzed for ^{32}P incorporation as described in Section 2. The data represent the mean value ± S.E.M., $n=6$. B: *Torpedo* electric organ cytosolic proteins (1 mg) were diluted into Lysis Buffer and incubated with glutathione-agarose containing 20 μg of either Fyn unique domain-GST fusion (Fyn-GST), Fyk unique domain-GST fusion (Fyk-GST) or GST backbone (GST) protein as described in Section 2. The bound proteins and 70 μg of cytosol (Cytosol) were analyzed by 15% SDS-PAGE and Western blotting using anti-Tctex-1 antibody. Arrow indicates the position of Tctex.

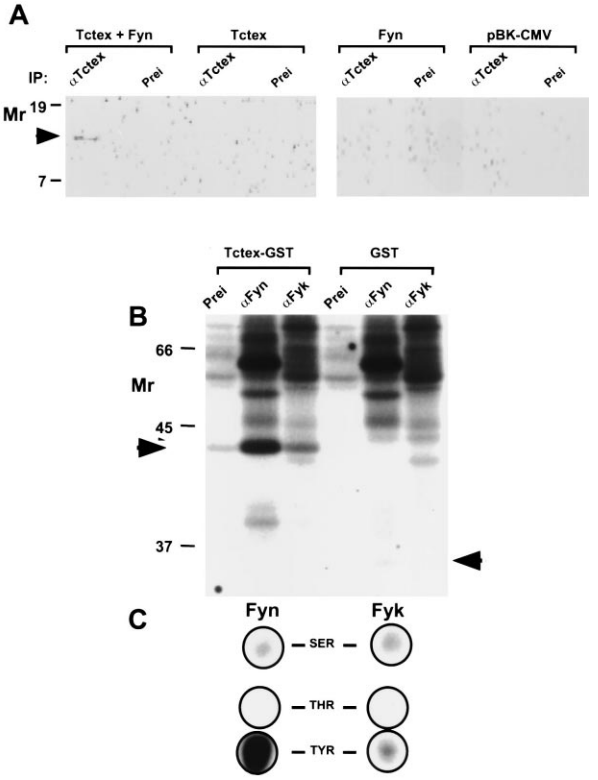

Fig. 5. Phosphorylation of Tctex-1 by Fyn. A: QT-6 fibroblasts were transfected with Tctex-1+Fyn, Tctex-1, or Fyn pBK-CMV expression constructs or empty pBK-CMV as indicated. The cellular proteins from two 100-mm dishes of transfected cells were solubilized, immunoprecipitated with anti-Tctex-1 (αTctex) or preimmune (Prei) serum and the immunoprecipitates analyzed by Western blotting using an anti-phosphotyrosine antibody as described in Section 2. Arrow indicates the position of Tctex-1. B: *Torpedo* electric organ postsynaptic membranes representing 500 μg of membrane protein were solubilized and immunoprecipitated with preimmune (Prei), anti-Fyn (αFyn), or anti-Fyk (αFyk) serum as described in Section 2. The immunoprecipitates were incubated with 2 μg of either Tctex-1-GST fusion (Tctex) or GST backbone (GST) protein and [γ-^{32}P]ATP under phosphorylating conditions for 30 min at 30°C as described in Section 2. Phosphorylation was analyzed by SDS-PAGE and autoradiography. Arrows indicate the positions of the Tctex-1-GST fusion and GST backbone proteins. C: Phosphorylated Tctex-1-GST fusion protein from B, was subjected to phosphoamino acid analysis as described in Section 2. The positions of phosphotyrosine (TYR), phosphothreonine (THR), and phosphoserine (SER) are indicated.

postsynaptic membranes, were incubated with Tctex-GST fusion or GST backbone proteins and [γ-^{32}P]ATP under phosphorylating conditions. Fyn was effective at phosphorylating the Tctex-GST fusion protein (Fig. 5B). The phosphorylation was very specific for Fyn since phosphorylation was not observed when the Tctex-GST fusion protein was incubated with immunoprecipitated Fyk or the preimmune precipitate. The site for phosphorylation occurred within the Tctex-1 sequence of the fusion protein because the GST backbone protein was not phosphorylated by Fyn (Fig. 5B). In addition, the phosphorylation occurred on tyrosine residue(s) as established by phosphoamino acid analysis (Fig. 5C). These data demonstrated that Tctex-1 could function as a specific substrate for Fyn in vitro and indicated that phosphorylation of Tctex-1 in vivo as shown in Fig. 5A was due to a direct phosphorylation of the microtubule protein by Fyn.

4. Discussion

The goal of this study was to investigate the synaptic function of Src kinases by characterizing postsynaptic substrates and/or regulators of Fyn. To do so, the identities of endplate components that interact with the kinase were sought. Using the unique domain of Fyn to probe a *Torpedo* electric organ library, a 13-kDa protein homologous to human Tctex-1 was cloned. Several lines of evidence support an interaction between Tctex-1 and Fyn in *Torpedo*. First, the method with which Tctex-1 was cloned from electric organ, the yeast two-hybrid system, is dependent on protein-protein interaction. In addition, others have also identified Tctex-1 as a binding partner for Fyn using the yeast two hybrid system, although the domain of Fyn involved and the relevance for the Tctex-1/Fyn interaction were not investigated [28]. In *Torpedo*, Tctex-1 was coexpressed with Fyn in the same tissues and subcellular fractions: electric organ, skeletal muscle, brain, and membranes, respectively. In addition, a Src class kinase was detected in the dynein complex of which Tctex-1 is a component. In vitro, the Tctex-1 fusion protein bound a protein tyrosine kinase activity and Tctex-1 bound specifically to the Fyn unique domain fusion protein. We were unable to show coimmunoprecipitation of Tctex-1 and Fyn from *Torpedo* electric organ due to insensitivity of the anti-Tctex-1 antibodies and/or low abundance of Tctex-1. However, the cloning based on protein-protein interaction, coexpression and colocalization in *Torpedo*, detection of a Src class kinase associated with the dynein complex, and in vitro binding provide evidence that Fyn and Tctex-1 form a complex.

An abundance of cytoskeletal proteins has been implicated in synapse formation and structure at the NMJ (for review see [29]). Tctex-1 is a light chain of the cytoplasmic and flagellar forms of the microtubule molecular motor protein dynein [11,21]. *Torpedo* Tctex-1, like mouse brain Tctex-1, was complexed with the 74-kDa intermediate chain of dynein demonstrating *Torpedo* Tctex-1 to be a component of dynein. Cytoplasmic dyneins produce force for retrograde transport along microtubules away from the plasma membrane and have been implicated in retrograde transport in axons [25,26,30–32]. In addition, several reports indicate the importance of microtubules in synaptic structure and function. For example, in the brain, glycine receptors are localized at inhibitory synapses via gephyrin-mediated anchoring to microtubules (for review see [33]). At the NMJ, cold stable and acetylated microtubules accumulate subsynaptically [34] and in the *Torpedo* electrocyte, microtubules converge towards the troughs of the postsynaptic folds [35]. The cytoskeletal protein Tctex-1 appears to have a synaptic role. Tctex-1 has been identified as a binding partner for Fyn in brain consistent with a role for the interaction of Tctex-1 and Fyn in synaptic function in the central nervous system [28]. In *Torpedo*, the relative abundance of Tctex-1 in electric organ and muscle as well as its presence in postsynaptic membranes and association with the AChR were consistent with a postsynaptic function at the NMJ. The reported accumulation of microtubules postsynaptically together with the established function of dynein in retrograde transport, suggested that a role for Tctex-1 at the postsynaptic membrane of the NMJ may be to translocate endplate components along microtubules.

Regulation of dynein by phosphorylation has been shown by previous studies. Both the 530-kDa heavy chain and the 74-kDa intermediate chain of dynein are phosphorylated on serine residues [16,36]. Phosphorylation of the heavy chain correlates with a dissociation of dynein from the lysosomes into a soluble pool with a diffuse distribution [36]. In addition, the heavy chain of inactive dynein being passively transported anterogradely is hypophosphorylated compared to active cellular dynein suggesting that serine phosphorylation regulates the functional activity of dynein [16]. Thus, phosphorylation appears to control dynein distribution and activity. Src-class protein tyrosine kinases regulate cytoskeletal function in several systems including neurite outgrowth at axonal growth cones [37]. The sequence of *Torpedo* Tctex-1 predicted a phosphorylation site at Tyr^4 of this cytoskeletal element (Fig. 1B). Human Tctex-1 also has a tyrosine at position four which is in a good context for phosphorylation: Glu-Asp-Tyr [20]. The results presented here demonstrated that Tctex-1 could be phosphorylated on tyrosine residues in vivo when coexpressed with Fyn as well as in vitro upon incubation with immunoprecipitated Fyn. These data in conjunction with the codistribution of Tctex-1 and Fyn in the same tissues and at the subcellular level as well as complex formation between the microtubule protein and the kinase suggested that tyrosine phosphorylation of Tctex-1 by Fyn may be an additional posttranslational mechanism for regulating dynein.

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References

[1] Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987) Annu. Rev. Biochem. 56, 567–613.
[2] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[3] Wagner, K.R., Mei, L. and Huganir, R.L. (1991) Curr. Opin. Neurobiol. 1, 65–73.
[4] Lai, C. and Lemke, G. (1991) Neuron 6, 691–704.
[5] Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S. and Fischbach, G.D. (1993) Cell 72, 801–815.
[6] Wallace, B.G., Qu, Z. and Huganir, R.L. (1991) Neuron 6, 869–878.
[7] Hopfield, J.F., Tank, D.W., Greengard, P. and Huganir, R.L. (1988) Nature (London) 336, 677–680.
[8] Swope, S.L. and Huganir, R.L. (1993) J. Biol. Chem. 268, 25152–25161.
[9] Swope, S.L. and Huganir, R.L. (1994) J. Biol. Chem. 269, 29817–29824.
[10] Chevray, P.M. and Nathans, D. (1992) Proc. Natl. Acad. Sci. USA 89, 5789–5793.
[11] King, S.M., Dillman III, J.F., Benahski, S.E., Lye, R.J., Patel-King, R.S. and Pfister, K.K. (1996) J. Biol. Chem. 271, 32281–32287.
[12] Huganir, R.L., Miles, K. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA 81, 6968–6972.
[13] Moscovici, C., Moscovici, M.G., Jimenez, H., Lai, M.M., Hayman, M.J. and Vogt, P.K. (1977) Proc. Natl. Acad. Sci. USA 11, 95–103.
[14] Gillespie, S.K., Balasubramanian, S., Fung, E.T. and Huganir, R.L. (1996) Neuron 16, 953–962.
[15] Kingston, R.E. (1997) in: Current Protocols in Molecular Biology (Chanda, V.B., Ed.) pp. 9.0.1–9.0.5, Wiley, New York, NY.

[16] Dillman III, J.F. and Pfister, K.K. (1994) J. Cell Biol. 127, 1671–1681.
[17] Peng, H.B. and Froehner, S.C. (1985) J. Cell Biol. 100, 1698–1705.
[18] Froehner, S.C., Murnane, A.A., Tobler, M., Peng, H.B. and Sealock, R. (1987) J. Cell Biol. 104, 1633–1646.
[19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
[20] Watanabe, T.K., Fujiwara, T., Shimizu, F., Okuno, S., Takahashi, E., Nakamura, Y. and Hirai, Y. (1996) Cytogenet. Cell Genet. 73, 153–156.
[21] Harrison, A., Olds-Clarke, P. and King, S.M. (1998) J. Cell Biol. 140, 1137–1147.
[22] Lader, E., Ha, H.S., O'Neill, M., Artzt, K. and Bennett, D. (1989) Cell 58, 969–979.
[23] Sobel, A., Weber, M. and Changeux, J.P. (1977) Eur. J. Biochem. (Germany) 80, 215–224.
[24] Qu, Z.C., Moritz, E. and Huganir, R.L. (1990) Neuron 4, 367–378.
[25] Brady, S.T. and Sperry, A.O. (1995) Curr. Opin. Neurobiol. 5, 551–558.
[26] Vallee, R.B. and Sheetz, M.P. (1996) Science 271, 1539–1544.
[27] Wagner, K., Edison, K., Heginbotham, L., Post, M., Huganir, R.L. and Czernik, A. (1991) J. Biol. Chem. 266, 23784–23789.
[28] Kai, N., Mishina, M. and Yagi, T. (1997) J. Neurosci. Res. 48, 407–424.
[29] Froehner, S.C. (1991) J. Cell Biol. 114, 1–7.
[30] Schnapp, B.J. and Reese, T.S. (1989) Proc. Natl. Acad. Sci. USA 86, 1548–1552.
[31] Hirokawa, N. (1998) Science 279, 519–526.
[32] Paschal, B.M. and Vallee, R.B. (1987) Nature 330, 181–183.
[33] Kirsch, J., Meyer, G. and Betz, H. (1996) Mol. Cell. Neurosci. 8, 93–98.
[34] Jasmin, B.J., Changeux, J.-P. and Cartaud, J. (1990) Nature 344, 673–675.
[35] Jasmin, B.J., Changeux, J.-P. and Cartaud, J. (1991) Neuroscience 43, 151–162.
[36] Lin, S.X.H., Ferro, K.L. and Collins, C.A. (1994) J. Cell Biol. 127, 1009–1019.
[37] Helmke, S. and Pfenninger, K.H. (1995) Cell Motil. Cytoskeleton 30, 194–207.