Identification of a *Torpedo* homolog of Sam68 that interacts with the synapse organizing protein rapsyn

Eric T. Fung, Anthony Lanahan, Paul Worley, Richard L. Huganir*

Department of Neuroscience, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, 904A PCTB, 725 North Wolfe Street, Baltimore, MD 21205, USA

Received 6 July 1998; received in revised form 21 August 1998

Abstract Nicotinic acetylcholine receptors (nAChRs) are initially expressed diffusely on the surface of myotubes and, in response to neuronally derived factors, cluster at the endplate to a final concentration of approximately 10 000/μm^2. The synaptic peripheral membrane protein rapsyn has been shown to mediate clustering of nAChRs in several systems. Here we describe the use of the yeast two-hybrid system to identify proteins that can interact with rapsyn. One of the clones we have identified is a *Torpedo californica* homolog of the Src-associated in mitosis protein (Sam68). We further show that Sam68, like rapsyn, is localized at the neuromuscular junction.

Key words: Neuromuscular junction; Rapsyn; Sam68

1. Introduction

The formation of a mature synapse is a complex process that requires pre-synaptic cues as well as post-synaptic modification and re-arrangement. Study of one synapse in particular, the neuromuscular junction, has revealed much about the processes underlying synaptogenesis, where it has been shown that receptors are initially expressed diffusely on the cell surface and subsequently cluster at the endplate (reviewed in [1,2]). Neuronally derived molecules such as agrin acts as the principal signal for the concentration of nicotinic acetylcholine receptor (nAChR) at the post-synaptic membrane (reviewed in [3,4]). In addition, a neuronally derived protein with acetylcholine receptor-inducing activity (ARIA) acts to increase the local transcription of mRNAs encoding nAChR subunits [5].

Although the initial signals for receptor clustering come principally from the motoneuron, a complex set of structural and transcriptional events must occur in the post-synaptic cell. The post-synaptic membrane is filled with invaginations termed junctional folds; nAChRs are segregated to the crests of the folds while sodium channels are limited to the depths of the folds. Transcripts for the subunits of the nAChR as well as those for acetylcholinesterase, NCAM, rapsyn, and s-laminin are concentrated at the neuromuscular junction [6–8]. In the case of the receptor subunits, the concentration of mRNAs is, at least in part, due to increased transcription by subsynaptic nuclei and relies on promoters upstream of the genes encoding the subunits.

In addition to mRNAs, a number of proteins have been observed to be enriched at the neuromuscular junction, including a β1 integrin, spectrin, and rapsyn [2]. Rapsyn, in particular, has sparked great interest because of its ability to form clusters when expressed in heterologous systems. For example, when expressed in oocytes [9] or in fibroblasts [10], rapsyn spontaneously forms clusters and, when co-expressed with subunits of the nAChR, the receptor co-clusters with rapsyn. Rapsyn has been cloned, and its primary sequence has revealed features that may be important for its function [11]. It is myristoylated, and mutation of the myristoylation site prevents the protein from reaching the plasma membrane [12]. The N-terminal half is notable for a leucine zipper while the C-terminus contains a zinc finger that has been shown to bind zinc. Mutation of two histidines that impair zinc binding impairs the ability of rapsyn protein to form large clusters; instead, it forms micro-clusters on the cell surface when expressed in oocytes [13]. More recently, rapsyn has been noted to contain eight tetratrichopeptide repeats, some of which appear to mediate self-association while others play a critical role in the clustering of acetylcholine receptor subunits [14,15]. Knockout of the gene for rapsyn has been shown to be lethal. These mutant mice fail to cluster nAChR as well as other synaptic proteins, suggesting that rapsyn plays a key role in the general process of clustering [16].

Despite the wealth of information thus far attained, the mechanism by which rapsyn works is still unknown. A first step toward understanding how rapsyn functions requires the identification of associated proteins, but co-immunoprecipitation studies have failed to identify interacting proteins. Two previous studies have used chemical cross-linking to show that rapsyn binds subunits of the nAChR, albeit different results were attained [17,18], and using a gel overlay assay, Walker et al. have shown that actin could bind immobilized rapsyn [19]. More recently, it has been shown that rapsyn and β-dystroglycan can be cross-linked to each other and associate in a variety of in vitro assays [20]. To identify other proteins that can bind rapsyn, we have used the yeast two-hybrid system. Using full-length rapsyn as a bait, we screened a library made from the electric organ of the *Torpedo californica*. We report here the identification and characterization of one of the interacting clones, TSam68, a homolog of the mammalian Sam68. Sam68 was originally identified as a protein that was highly tyrosine phosphorylated during mitosis [21] and that has been shown to associate with a diverse array of signal transduction molecules [22].

*Corresponding author. Fax: (1) (410) 955-0877.
E-mail: rick.huganir@qmail.bs.jhu.edu

Abbreviations: nAChR, nicotinic acetylcholine receptor; DABCO, 1,4-diazabicyclo[2,2,2]-octane; SAM68, src-associated in mitosis; ARIA, acetylcholine receptor-inducing activity; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; PKC, protein kinase C; SDS, sodium dodecyl sulfate

PII: S0014-5793(98)01151-X

2. Materials and methods

2.1. Materials

All molecular biology reagents were from New England Biolabs unless specifically stated otherwise. Antibodies used in this study were a monoclonal anti-rapsyn 1234 (gift of Dr. John Merlie) and a monoclonal anti-Sam68 (Transduction Laboratories).

2.2. Yeast strains and methods

The yeast strain Y190 was kindly provided by Dr. Steve Elledge (Baylor University). The full-length cDNA encoding rapsyn was cut out of the vector pGW1-CMV, blunt-ended with Klenow, ligated to *Bgl*II linkers, cut with *Bgl*II, and ligated into the yeast expression vector pPC97 [23]. This placed full-length rapsyn in-frame with the DNA binding domain of the GAL4 transcriptional activator. A cDNA library was made from the electric organ of the *Torpedo californica* using the Superscript cDNA library synthesis kit (Gibco) and subcloned into the yeast expression vector pPC86. This fused *Torpedo* cDNAs with the activation domain of GAL4. Transformation of yeast with the rapsyn clone was performed using the lithium acetate/PEG method. Transformation of the library into yeast expressing rapsyn-GAL4 binding domain was performed using a variation of the lithium acetate/PEG method. Briefly, yeast were grown to an $OD_{600}=\sim 1.0$, pelleted, and washed with water. They were then incubated in 100 mM lithium acetate/10 mM Tris pH 7.5/1 mM EDTA/ 1 M sorbital (LiSorb) at 30°C for 30 min, pelleted, and resuspended in 2.5 ml of LiSorb. To this, 50 μg of library+800 μg of carrier DNA in a final volume of 3 ml of LiSorb was added followed by 24 ml of 100 mM lithium acetate/10 mM Tris pH 7.5/1 mM EDTA/ 40% PEG. Yeast were incubated for 30 min at 30°C and then for 15 min at 42°C. 100 ml of leu−trp−his− medium was added and yeast were incubated for 3 h at 30°C. Yeast were pelleted, resuspended in leu−trp−his− medium and plated on leu−trp−his− plates containing 50 mM 3-aminotriazole and incubated at 30°C. Colonies that appeared after 5 days were re-streaked and tested for activation of lacZ. Plasmids were isolated from yeast using glass beads and transformed into bacteria and sequenced. PCR primers were used to amplify the coding regions of these constructs and PCR products were subcloned into pPC97.

2.3. Isolation of full-length clone

A *Torpedo* electric organ oligo-dT primed cDNA library was constructed and cloned into lambda-ZAP II. Five million clones were screened using the *Sal*I-*Pst*I fragment of the *Torpedo* clone, yielding 20 positives.

2.4. Tissue isolation

Adult rat tissues or *Torpedo californica* tissues were isolated, homogenized in buffer (10 mM Tris pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 10 U/ml aprotinin, 1 mM Na_3VO_4, 1 mM $(NH_4)_3MoO_4$, 1 mM NaF), and separated by SDS-PAGE.

2.5. Fusion protein binding

To construct the TSAM68 fusion protein, the plasmid containing the TSAM68 clone was cut with *Sal*I and *Not*I, which released the insert. Deletion mutants were constructed as follows. The *Sal*I-*Xmn*I (encoding amino acids 1–42) fragment was cut out and ligated into the *Sal*I-*Eco*RV sites of Bluescript. The *Sal*I-*Pst*I (encoding amino acids 1–264) fragment was cut out and ligated into the *Sal*I-*Pst*I sites of Bluescript. The inserts were released using *Sal*I and *Not*I and ligated into pGEX4T-2 (Pharmacia), and used to transform TOP 2 cells (Stratagene) or BL21(DE3). Fusion protein was produced and purified according to the manufacturer's directions. *Torpedo* electric organ was fractionated as described [24]. Alkaline extracts of receptor-rich membranes were prepared by diluting in water to a final concentration of 1 mg/ml, adding 100 mM NaOH to pH 11, and incubating on ice for 30 min. The supernatant of a $100\,000\times g$ spin was neutralized with 100 mM HCl, diluted in 5 volumes of binding buffer, and incubated with 4 μg of GST-agarose or 2 μg of TSAM68-GST-agarose fusion proteins for 3 h at 4°C. Beads were washed five times in binding buffer. After the last wash, 50 μl of 3× gel sample buffer was added, and loaded onto a 10% SDS-acrylamide gel.

2.6. Generation of anti-TSAM68 antibody

GST-TSAM68 was purified as above and used to immunize rabbits.

2.7. Immunoblotting

Proteins that had been separated on SDS-PAGE gels under reducing conditions were transferred to Immobilon-P membranes (Millipore) and blocked in 0.5% non-fat milk/0.1% Tween/TBS (blocking buffer) for 1 h. Membranes were incubated in primary antibody diluted in blocking buffer for 1 h, washed with blocking buffer, incubated in peroxidase-conjugated anti-mouse or anti-rabbit IgG for 1 h, washed extensively with blocking buffer, washed with TBS, and then visualized with enhanced chemiluminescence. All incubations were performed at room temperature.

2.8. Immunohistochemistry

Skeletal muscle was dissected, frozen in cold isopentane, and embedded in OCT. 8 μm sections were cut and mounted onto subbed

A

```
   ctgaggcggacgacagttagtgtgcgcggcgaaacgaagaacgcagaccaaggagag
ATGGCGACCGATAAATACCTTCCCGAGTTGATGGCTGAAAGGGACAGCCTGGATCCCTCC    60
M  A  T  D  K  Y  L  P  E  L  M  A  E  R  D  S  L  D  P  S     20
TTCGTACACGGTTCTCGGCTGCTGTCGGCAGAAATTGAAAAGGTTCAGAAAGGAGAGAAG   120
F  V  H  G  S  R  L  L  S  A  E  I  E  K  V  Q  K  G  E  K     40
AAGAAAGAAGAGGAAAAGAGATTTCTTGATGTTCTGACACAGAAAAATATCAAGCTCTCT   180
K  K  E  E  E  K  R  F  L  D  V  L  T  Q  K  N  I  K  L  S     60
GAGCGATGCCTGATACCGGTTTCACAGTTCCCAAAGTTCAACTTTGTTGGGAAACTACTT   240
E  R  C  L  I  P  V  S  Q  F  P  K  F  N  F  V  G  K  L  L     80
GGACCAAGAGGAAATTCATTGAAGCGACTACAAGAGGACACTGGCACAAAAATGTCCATC   300
G  P  R  G  N  S  L  K  R  L  Q  E  D  T  G  T  K  M  S  I     100
CTTGGGAAAGGCTCAATGAGAGATAAGGCCAAGGAAGATGAACTCCGTAAAGGAGGAGAA   360
L  G  K  G  S  M  R  D  K  A  K  E  D  E  L  R  K  G  G  E     120
GCTAAGTACGCACACTTGAACAATGATCTGCATGTACTTGTTGAAGTCTTTGGACCTCCA   420
A  K  Y  A  H  L  N  N  D  L  H  V  L  V  E  V  F  G  P  P     140
ACTGAAGCCTATTCACGAATGTACCATGCAATGGAGGAGGTTAAAAAATTTCTTATACCC   480
T  E  A  Y  S  R  M  Y  H  A  M  E  E  V  K  K  F  L  I  P     160
GACTACAATGATGAAATAAGGGAGGAGCAGCTAACTGAGCTGGCATACTTGAATGGTGGA   540
D  Y  N  D  E  I  R  E  E  Q  L  T  E  L  A  Y  L  N  G  G     180
CAAGATGCATCCCGTGGGAGAGGTGTACGTGCGCGAGGTACTCTACCTGTACCACTTGCG   600
Q  D  A  S  R  G  R  G  V  R  A  R  G  T  L  P  V  P  L  A     200
AGTGTGTTCATTTCCAGGGGTCGTGGTGTCGCACCACTTCCGCCCTCCCCTCAGTACGGA   660
S  V  F  I  S  R  G  R  G  V  A  P  L  P  P  S  P  Q  Y  G     220
CCACGAGGTGCAGTGACACGTGGAGCATCTGTGAGGGGAGCTCCAGGAGGTCGCAGAGAA   720
P  R  G  A  V  T  R  G  A  S  V  R  G  A  P  G  G  R  R  E     240
GTGACAAGTGGACGTGGTGTCACCCCAGGACAGAGAGGTGCCCCAGCGCCTCGTGCTCGA   780
V  T  S  G  R  G  V  T  P  G  Q  R  G  A  P  A  P  R  A  R     260
GGGACAACTGCAGTCTACAGACAGGCACTGCACCCGCCGCCTGCTCAGGAGAGCTATGAT   840
G  T  T  A  V  Y  R  Q  A  L  H  P  P  P  A  Q  E  S  Y  D     280
GAATACGGCTATGATGATGGGTATTCGGAGCACAATTATGATGATAATTATGAAAACTATTAT  900
E  Y  G  Y  D  D  G  Y  S  E  H  N  Y  D  N  Y  E  N  Y  Y     300
GATCAAGCCCAGGGGGACGCAGAATATTTTGACTATGGGCATGGTGAAGTGCAGGACACT   960
D  Q  A  Q  G  D  A  E  Y  F  D  Y  G  H  G  E  V  Q  D  T     320
TACGATGATTATGGCCAGGAAGATTGGAATGCAGGACGAACAAGCAGGAAGGCACCAGCA  1020
Y  D  D  Y  G  Q  E  D  W  N  A  G  R  T  S  R  K  A  P  A     340
AGGCCACTCAAGGGAATGTACAGAGATCATCCCTATGGGCGATACTAA              1065
R  P  L  K  G  M  Y  R  D  H  P  Y  G  R  Y  *                 355
```

B

```
Mouse     MQRRDDPASRLTRSSGRSCSKDPSGAHPSVRLTPSRPSPLPHRPR
Mouse     GGGGGPRGGARASPATQPPPLLPPSTPGPDATVVGSAPTLPLLPP

Torpedo             MATDKYLPELMAERDSLDPSFVHGSRLLSAEIEKVQK
                       ||  ||||||||||||||  |   ||| |||||||
Mouse     SATAAVKMEPENKYPPELMAEKDSLDPSFTHAMQLLSVEIEKIQK

Torpedo   -GEKKKEEEKRFLDVLTQKNIKLSERCLIPVSQFPKFNFVGKLLG
           ||  ||   |   |||  | ||  ||  ||  |||| | ||||||||||
Mouse     KGESKKDDEENYLDLFSHKNMKLKERVLIPVKQYPKFNFVGKILG

Torpedo   PRGNSLKRLQEDTGTKMSILGKGSMRDKAKEDELRKGGEAKYAHL
          |||||||||||||| | ||||||||||||||||||||||| |||||
Mouse     PQGNTIKRLQEETGAKISVLGKGSMRDKAKEEELRKGGDPKYAHL

Torpedo   NNDLHVLVEVFGPPTEAYSRMYHAMEEVKKFLIPDYNDEIREEQL
          | |||| ||||||| |||| | ||||||||||||  |||  ||
Mouse     NMDLHVFIEVFGPPCEAYALMAHAMEEVKKFLVPDMMDDICQEQF

Torpedo   TELAYLNGGQDASRGRGVRARGTLPVPLASVFISRGRGVAPLPPS
           |||||||  | ||||||  ||    |   |   || |
Mouse     LELSYLNGVPEPSRGRGVSVRGRGAAPPPPVPRGRGVGPPRGALV

Torpedo   PQYGPRGAVTRGASV-RGAPGGRREVTSGRGVTPGQRGAPAPRAR
                ||||||||||  || |                ||||  ||||
Mouse     RGTPVRGSITRGATVTRGVPPPPTV----------RGAPTPRAR

Torpedo   GTTAVYRQALHPPPAQESYDEYGYDDGYSEHNYDNYENYYDQAQG
           |  | |  | | || |||||||||| |||  || || || ||||
Mouse     -TAGIQRIPLPPTPAPETYEDYGYDDTYAEQSYEGYEGYYSQSQG

Torpedo   DAEYFDYGHGEVQDTYDDYGQEDWNAGRTSRKAP-ARPLKGMYR
          |||| |||||||||||| |||||||  | | ||| |||||| ||
Mouse     ESEYYDYGHGELQDSYEAYGQDDWNGTRPSLKAPPARPVKGAYR

Torpedo   HPYGRY
          ||||||
Mouse     HPYGRY
```

Fig. 1. Sequence and alignment of TSam68 with mouse Sam68. A: The nucleotide sequence of TSAM68 and its amino acid translation are shown. Underlined are two potential start methionines. In addition, the single proline-rich stretch of TSam68 is in bold. B: Alignment of TSam68 with mammalian Sam68. Identical and homologous amino acids are indicated by vertical dashes. Note conservation in the amino- and carboxy-termini of TSam68 with the mouse Sam68 but divergence in the central region (amino acids 190–251).

slides. Sections were fixed in PBS containing 1% paraformaldehyde, 100 mM lysine, 10 mM sodium *meta*-periodate, 0.1% saponin at room temperature for 20 min or in PBS containing 4% paraformaldehyde. They were then permeabilized with PBS containing 1% Triton for 10 min at room temperature. To reduce non-specific fluorescence, sections were incubated with PBS containing 10% normal goat serum for greater than 1 h at room temperature, and then incubated with primary antibody diluted in PBS containing 3% normal goat serum overnight at 4°C. Sections were then washed with PBS and then incubated with biotinylated anti-mouse (Vector) at room temperature for 1 h. Sections were washed with PBS and then incubated with FITC-streptavidin (Vector) and rhodamine α-bungarotoxin (Molecular Probes) for 1 h at room temperature. Sections were then washed with PBS and mounted using Permafluor containing 2.5% DABCO.

3. Results

A screen of the *Torpedo* electric organ library encompassing ~1.0 million clones using rapsyn as a bait yielded 16 positives, as assayed by histidine auxotrophy and activation of β-galactosidase. Nine of these clones encoded the *Torpedo* type III intermediate filament [25]. Three encoded two independent clones of a *Torpedo* homolog of Sam68 (TSam68). These clones were used to screen a *Torpedo* electric organ cDNA library, and the sequence of longest clone is shown here (Fig. 1A). Two methionines (underlined) fulfill requirements

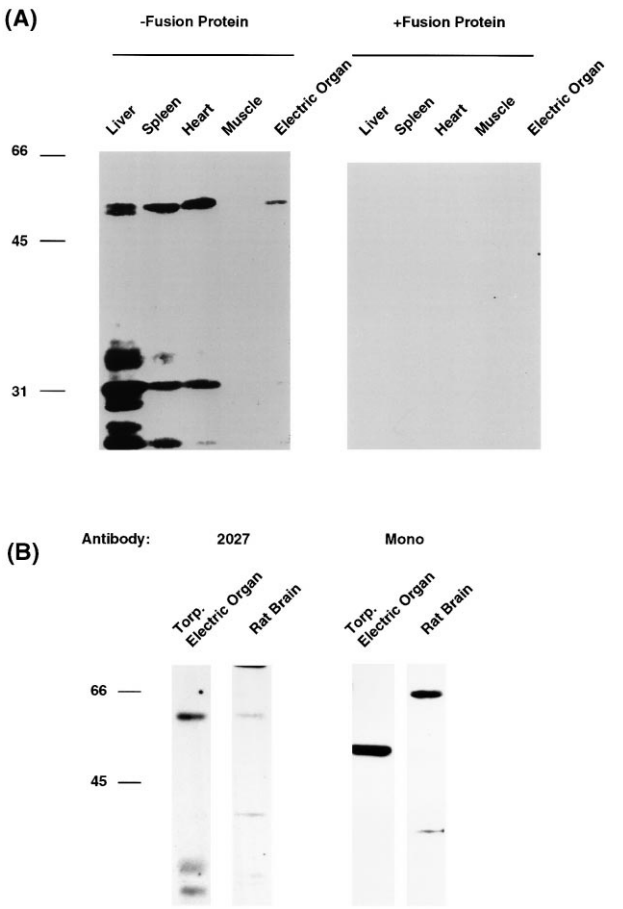

Fig. 2. Western blot analysis of TSam68 and Sam68. A polyclonal antibody (2027) was generated against a fusion protein encompassing full-length TSam68. The antibody recognizes bands in the 50 kDa range, which can be blocked by pre-incubation with fusion protein (A). Both 2027 and a commercial monoclonal antibody recognize similar bands in the *Torpedo* electric organ and a 68 kDa band in rat brain (B).

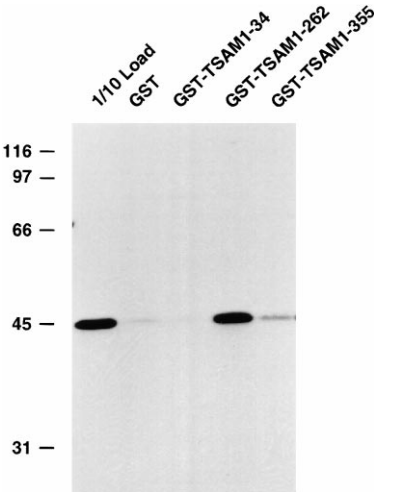

Fig. 3. Binding of rapsyn to immobilized TSam68. GST-fusion proteins of TSam68 were constructed and incubated with alkaline extracts of receptor-rich *Torpedo* electric organ membranes. Immunoblot reveals that alkaline extracted rapsyn binds to both full-length and a C-terminal deletion mutant of TSam68.

for a consensus start site [26]. The sequence of TSam68 has regions of high homology to mammalian Sam68, including conservation of a number of arginine-glycine repeats and a carboxy-terminus rich in consensus tyrosine phosphorylation sites (Fig. 1B). Arginine-glycine repeats have been implicated in RNA binding, while tyrosine phosphorylation sites may indicate SH2-binding domains and/or sites of functional modulation. In the case of SAM68, tyrosine phosphorylation has been shown to regulate its interaction with RNA and with a variety of signaling proteins [22,27]. TSam68 differs from its mammalian counterpart in several significant aspects. Most notably, the cDNA encodes a smaller protein, lacking the first 100 amino acids which is confirmed by immunoblotting *Torpedo* tissues using antibodies directed against both the *Torpedo* and the human clones (see below). Another difference between TSam68 and its mammalian counterpart is that the *Torpedo* clone includes a 10 amino acid insert not present in the mammalian clone. Finally, it lacks four proline-rich regions and instead contains a single proline-rich stretch (in bold) that is most similar to the fifth proline stretch of the mammalian clone.

To examine the expression of the protein in *Torpedo* tissues, we performed a multiple tissue Western blot using an antibody produced against the full-length TSam68 fusion protein (2027). Fig. 2A shows that TSam68, like its mammalian counterpart, is widely expressed but has a mobility corresponding to 50 kDa in accordance with its shorter primary sequence. The lower molecular weight bands seen in the liver, spleen, and heart samples probably represent degradation products, since those bands, like the 50 kDa bands, could be blocked by pre-incubation of the antibody with the TSAM68 fusion protein. Both the polyclonal antibody and a commercial monoclonal antibody produced against the human Sam68 recognize a single band of identical mobility in *Torpedo* membranes with an apparent molecular mass of 50 kDa (Fig. 2B).

To test the interaction between rapsyn and TSam68 in vitro, we assessed the ability of GST-TSam68 to bind rapsyn. Alkaline extracts of nAChR-rich membranes from the electric organ of the *Torpedo californica*. which are enriched in rapsyn

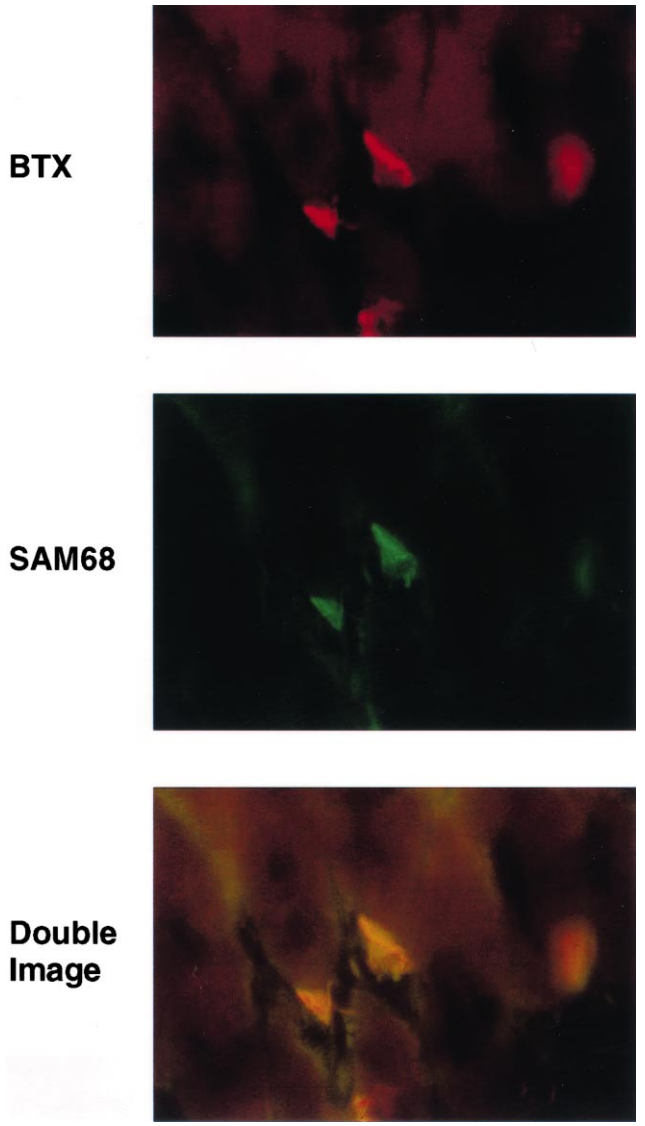

Fig. 4. Sam68 is localized at adult rat neuromuscular junctions. 10 μm thin section of adult rat skeletal muscle were labeled with rhodamine α-bungarotoxin to label neuromuscular junctions (top) and the monoclonal antibody to identify the distribution of Sam68 (middle).

were prepared and incubated with GST-TSam68 or GST backbone alone. As shown in Fig. 3, rapsyn bound the full-length TSam68 fusion protein but not the backbone. Moreover, deletion of the terminal 90 amino acids of TSam68 resulted in more efficient binding, suggesting that the carboxy-terminus may act as a negative regulatory domain. The amino-terminal 42 amino acids were not adequate for binding. Interestingly, although Triton extraction released an amount of rapsyn similar to that released by alkaline extraction, rapsyn did not bind to TSam68 fusion proteins under these conditions (data not shown). Co-immunoprecipitation of the complex from skeletal muscle and from *Torpedo* electric organ was attempted using both anti-rapsyn and anti-SAM68 antibodies, but no complex was identified. This may be due to the instability of this complex after solubilization and dilution of the electric organ.

To see if Sam68 was present at the adult neuromuscular junction, we stained sections of rat muscle with the monoclonal antibody against Sam68 and used α-bungarotoxin to identify endplates (Fig. 4). Staining demonstrated immunoreactivity that appeared diffusely along membranes and that was enriched at endplates. This staining pattern could be blocked with a GST fusion protein of the *Torpedo* clone (data not shown).

4. Discussion

In this study, we have identified a protein that interacts with the post-synaptic nicotinic acetylcholine receptor-associated rapsyn. This protein, the *Torpedo* homolog of Sam68, shares several features with its mammalian counterpart. This includes sequences compatible with RNA binding, a tyrosine-rich tail, and a proline-rich stretch suggestive of an SH3 domain-binding domain. The mammalian Sam68 was identified on the basis of being in a state of increased tyrosine phosphorylation during mitosis [21]. In addition, it has been shown to associate with c-Src via SH2 and SH3 domains during mitosis [28]. Sam68 can bind RNA in a phosphorylation-dependent manner [27], is a substrate for src-like kinases, and associates with signal transduction molecules such as Grb2 and phospholipase Cγ-1 [22]. A striking possibility is that Sam68 might serve to transduce signals from cell-surface receptors to the nucleus via the ras pathway, and, because of its many potential binding sites for a wide array of molecules, it might serve to integrate and/or modulate a complex set of signals.

The differences between the mammalian and *Torpedo* clones are intriguing. The N-terminal amino acids of the mammalian clone extend approximately 90 amino acids longer than the *Torpedo* clone. Included in this domain are a proline-rich region and a poly-glycine stretch. However, no function has been ascribed to this domain, nor is this proline-rich region required for SH3 domain binding [22]. While the mammalian SAM68 contains four additional proline-rich regions, the TSam68 contains a single one (PLPPSP) which corresponds most closely to the fifth proline-rich region of Sam68 (PLPPPAP) (also called P5). P5 has been shown to mediate the majority of binding of Sam68 with c-src [22], and both the *Torpedo* and mammalian form of P5 agree with consensus src SH3 binding sites [29]. TSam68 binds to the SH3 domain of two src-like kinases, fyn and fyk (data not shown). Finally, TSam68 contains a 10 amino acid stretch not contained in Sam68. This insert lies in a distinctly non-conserved region of TSam68, in which the homology is 47%. Outside of this region, the homology is 78%. We were unable to identify longer *Torpedo* clones in several library screens, nor did we identify clones that lacked this insert. The possibility remains that mammalian alternatively spliced products exist that contain this insert but require more sensitive methods to be identified [30].

Since TSam68 interacts with rapsyn and is present at the neuromuscular junction, it may play a role in synapse formation. For example, it could participate in the transduction of signals generated by ARIA or agrin, since both processes require tyrosine phosphorylation of cell surface receptors and/or cytoplasmic proteins. ARIA increases AChR α-subunit mRNA expression via mechanisms that require tyrosine phosphorylation of its receptor (ErbB3/ErbB2) [31,32]. Agrin has been also shown to activate receptor tyrosine kinases. In

addition, agrin induces phosphorylation of several components, in the postsynaptic cell, including the nicotinic acetylcholine receptor, and this phosphorylation is critical for agrin's role in receptor clustering [33,34]. Sam68 and TSam68 may be involved in ARIA or agrin-mediated signal pathways via their interaction with src-like kinases, which are able to phosphorylate the nAChR [35]. Sam68 can bind to fyn [22] while the single polyproline region of TSam68 can bind the SH3 domain of *Torpedo* fyn (data not shown). Thus, it will be interesting to see if TSam68 can promote the phosphorylation of nAChR subunits.

While the possibilities for a signal transduction role of Sam68 in myotube differentiation are intriguing, one cannot ignore the potential structural role Sam68 might play. Its presence at adult neuromuscular junctions suggests that it has a role even once an endplate has been established. Since the structural role of rapsyn in the formation and maintenance of nAChR clusters has been well established, it would be intriguing to understand how Sam68 might participate in this process. One possibility is that Sam68 might serve to direct mRNA localization. A previous study [36] has shown that Sam68 could be identified at motile edges of fibroblasts, the same area in which high concentrations of β-actin mRNA was found. mRNAs for a number of structural proteins have been shown to be differentially targeted [37], including those for actin, vimentin, and muscle myosin. Their common denominator is that they quickly undergo self-assembly post-translationally, and hence targeting occurs at the mRNA level rather than at the protein level. It would not be surprising if rapsyn underwent similar, rapid self-assembly (e.g. through multimerization via its tetratrichopeptide repeats), and it has been shown that rapsyn mRNAs are concentrated at the neuromuscular junction [7,15]. The widespread expression of Sam68 makes a specific role in synapse formation unlikely. However, it is possible that rapsyn might confer some specificity to a Sam68-RNA interaction. Rapsyn has an N-terminal leucine zipper and a C-terminal zinc finger, which make it a candidate for interacting with either DNA or RNA. Thus, TSam68 and rapsyn may together act to localize and/or stabilize mRNAs at the endplate.

Acknowledgements: The authors would like to thank Carol Doherty for technical assistance, Julie Leegwater-Kim and Dr. Susan Gillespie for maintaining Sol8 and C2 cell lines, and Dr. Pierre Chevray for help with the yeast two-hybrid system.

References

[1] Hall, Z. and Sanes, J. (1993) Neuron/Cell 72, (Suppl.) 99–121.
[2] Froehner, S.C. (1993) Annu. Rev. Neurosci. 16, 347–368.
[3] Hoch, W., Campanelli, J.T. and Scheller, R.H. (1994) J. Cell. Biol. 126, 1–4.
[4] Bowe, M.A. and Fallon, J.R. (1995) Annu. Rev. Neurosci. 18, 443–462.
[5] Usdin, T.B. and Fischbach, G.D. (1986) J. Cell. Biol. 103, 493–507.
[6] Jennings, C.G. and Burden, S.J. (1993) Curr. Opin. Neurobiol. 3, 75–81.
[7] Moscoso, L.M., Merlie, J.P. and Sanes, J.R. (1995) Mol. Cell. Neurosci. 6, 80–89.
[8] Jasmin, B.J., Lee, R.K. and Rotundo, R.L. (1993) Neuron 11, 467–477.
[9] Froehner, S.C., Luetje, C.W., Scotland, P.B. and Patrick, J. (1990) Neuron 5, 403–410.
[10] Phillips, W.D., Kopta, C., Blount, P., Gardner, P.D., Steinbach, J.H. and Merlie, J.P. (1991) Science 251, 568–570.
[11] Phillips, W.D., Maimone, M.M. and Merlie, J.P. (1991) J. Cell Biol. 115, 1713–1723.
[12] Musil, L.S., Carr, C., Cohen, J.B. and Merlie, J.P. (1988) J. Cell Biol. 107, 1113–1121.
[13] Scotland, P.B., Colledge, M., Melnikova, I., Dai, Z. and Froehner, S.C. (1993) J. Cell Biol. 123, 719–728.
[14] Ponting, C.C. and Phillips, C. (1996) Biochem. J. 314, 1053–1054.
[15] Ramarao, M.K. and Cohen, J.B. (1998) Proc. Natl. Acad. Sci. USA 95, 4007–4012.
[16] Gautam, M., Noakes, P.G., Mudd, J., Nichol, M., Chu, G.C., Sanes, J.R. and Merlie, J.P. (1995) Nature 377, 232–236.
[17] Burden, S.J., DePalma, R.L. and Gottesman, G.S. (1983) Cell 35, 687–692.
[18] Shoji, H., Nomoto, H., Ohta, M. and Hayashi, K. (1992) Biochem. Int. 28, 1071–1077.
[19] Walker, J.H., Boustead, C.M. and Witzemann, V. (1984) EMBO J. 3, 2287–2290.
[20] Cartaud, A., Coutant, S., Petrucci, T.C. and Cartaud, J. (1998) J. Biol. Chem. 273, 11321–11326.
[21] Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M.F., Polakis, P. and McCormick, F. (1992) Cell 69, 551–558.
[22] Richard, S., Yu, D., Blumer, K.J., Hausladen, D., Olszowy, M.W., Connelly, P.A. and Shaw, A.S. (1995) Mol. Cell. Biol. 15, 186–197.
[23] Chevray, P.M. and Nathans, D. (1992) Proc. Natl. Acad. Sci. USA 89, 5789–5793.
[24] Huganir, R.L., Miles, K. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA 81, 6968–6972.
[25] Frail, D.E., Mudd, J. and Merlie, J.P. (1990) Nucleic Acids Res. 18, 1910.
[26] Kozak, M. (1991) J. Biol. Chem. 266, 19867–19870.
[27] Wang, L.L., Richard, S. and Shaw, A.S. (1995) J. Biol. Chem. 270, 2010–2013.
[28] Taylor, S.J. and Shalloway, D. (1994) Nature 368, 867–871.
[29] Van Seuningen, I., Ostrowski, J., Bustelo, X.R., Sleath, P.R. and Bomsztyk, K. (1995) J. Biol. Chem. 270, 26976–26985.
[30] Weng, Z., Rickles, R.J., Feng, S., Richard, S., Shaw, A.S., Schreiber, S.L. and Brugge, J.S. (1995) Mol. Cell. Biol. 15, 5627–5634.
[31] Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocque, B. and Schweighoffer, F. (1997) J. Biol. Chem. 272, 3129–3132.
[32] Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S. and Fischbach, G.D. (1993) Cell 72, 801–815.
[33] Altiok, N., Bessereau, J.L. and Changeux, J.P. (1995) EMBO J. 14, 4258–4266.
[34] Wallace, B.G., Qu, Z. and Huganir, R.L. (1991) Neuron 6, 869–878.
[35] Swope, S. and Huganir, R. (1994) J. Biol. Chem. 269, 29817–29824.
[36] Hill, M.A., Schedlich, L. and Gunning, P. (1994) J. Cell. Biol. 126, 1221–1229.
[37] Wilhelm, J.E. and Vale, R.D. (1993) J. Cell. Biol. 123, 269–274.