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Mapping of a dominant immunogenic region of synaptophysin, a major membrane protein of synaptic vesicles

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Synaptophysin is a major integral membrane protein of synaptic vesicles. Its transmembrane topology deduced from the cDNA sequence predicts 4 transmembrane regions and a carboxy-terminal cytoplasmic tail containing a characteristic pentapeptide repeat structure. The monoclonal antibody (mAb), SY38, binds to a cytoplasmic domain of synaptophysin. By using fusion proteins corresponding to truncated forms of the cytoplasmic tail, its epitope was located to a flexible segment in the center of the repeat structure. Four other mAbs (c7.1, c7.2, c7.3, c7.4) share the same epitope, which thus emerges as the major immunogenic region of this membrane protein.

Synaptophysin; Synaptic vesicle; Antigenic epitope; Site-directed mutagenesis; (Rat brain)

1. INTRODUCTION

Synaptic vesicles are organelles specialized for packaging, storing and releasing neurotransmitters. In presynaptic terminals different populations of these vesicles are found which differ in size and contents. Synaptophysin is a hexameric glycosylated protein of M_r 38000 which is localized in small clear vesicles (40–60 nm diameter) [1,2]. Its primary structure has been determined from cloned cDNA [3,4]. The polypeptide is predicted to span the membrane 4-times and has a long cytoplasmic tail with a novel type of repeat structure (fig.1). This transmembrane topology is supported by protease digestion and antibody binding data [3,5,6] and resembles that of connexins which form transmembrane channels allowing cellular coupling via gap junction structures. After incorporation into planar lipid bilayers, synaptophysin displays voltage-sensitive channel activity [7]. This membrane protein therefore is thought to mediate translocation of vesicular contents during packaging and/or release of neurotransmitter.

Synaptophysin was initially identified and localized by using a monoclonal antibody (mAb), SY38, which resulted from immunization with coated vesicles of bovine brain tissue [1]. Immunogold labelling showed that this antibody binds to a cytoplasmic domain of the membrane protein. Here, we have localized the epitope of mAb SY38 by using fusion proteins covering variable portions of the cytoplasmic tail region. Our data define a dominant immunogenic determinant of this abundant vesicle channel protein.

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Abbreviations: mAb, monoclonal antibody; SCY, synaptophysin cytoplasmic tail; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Fusion constructs*

To isolate the cDNA sequence corresponding to the C-terminal cytoplasmic tail of synaptophysin, an artificial *Eco*RI restriction site was introduced by site-directed mutagenesis after transmembrane region 4 (fig.1). The resulting *Eco*RI fragment, called SCY (*s*ynaptophysin *cy*toplasmic tail), was subcloned into the expression vector pEX34b [8].

The SCY fragment was also cloned into M13mp18 and used as a template for introducing stop codons (TAG) at 4 different positions by the method of Sayers et al. [9]. The resulting mutants (see fig.1) were recloned into pEX34b. All constructs were confirmed by DNA sequencing [10].

2.2. *Expression of fusion proteins*

Expression of fusion proteins in *E. coli* (Pop 2136) was induced by a 2 h incubation at 42°C, a condition which inactivates the temperature-sensitive cI-repressor of this strain [8]. Bacteria then were collected by centrifugation and solubilized in SDS sample buffer [11].

2.3. *Electrophoresis and immunoblotting*

SDS-polyacrylamide electrophoresis (SDS-PAGE) was performed according to Laemmli [11], and Western blotting using the method of Towbin et al. [12]. Polyclonal antibodies were visualized using alkaline-phosphatase conjugated anti-rabbit IgG (Promega), and monoclonal antibodies using alkaline phosphatase conjugated anti-mouse IgG (Promega).

3. RESULTS AND DISCUSSION

After introduction of an artificial *Eco*RI site, a fragment of the synaptophysin cDNA encoding the

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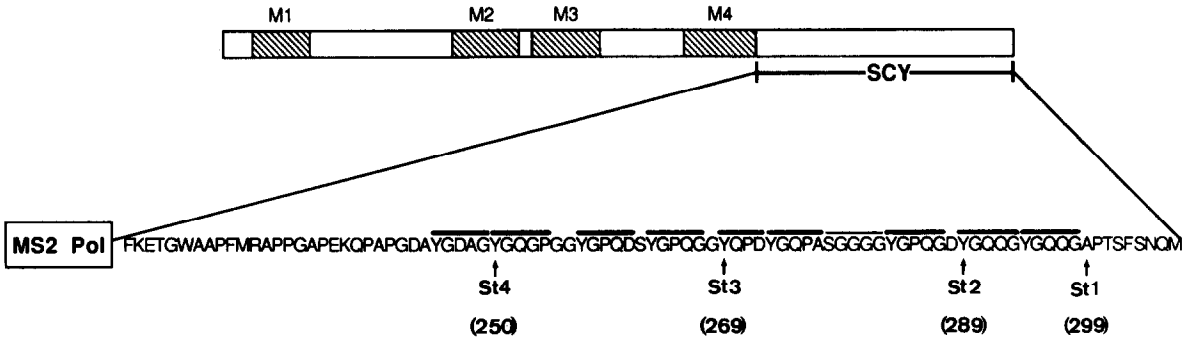

Fig.1. Construction of truncated MS2-SCY fusion proteins. An *Eco*RI site was introduced in the synaptophysin cDNA after the transmembrane regions (M1–M4; hatched boxes), and the SCY-fragment then subcloned into pEX34 to obtain a fusion construct with MS2 polymerase. Stop-codons introduced by oligonucleotide-directed mutagenesis are indicated by arrows. Pentapeptide repeats are overlined by bars; a thin bar indicates the degenerate repeat of the SY38 epitope. For amino acid numbering, see [3].

cytoplasmic tail region was subcloned into the expression vector pEX34. The resulting MS2 polymerase fusion protein (SCY-MS2) was recognized in Western blots by both the mAb SY38 [1] and a polyclonal antiserum raised against a purified synaptophysin preparation [3] (see fig.2, lane 5).

To obtain truncated forms of the cytoplasmic tail of synaptophysin, the SCY insert was cloned into M13mp18, and the single-stranded DNA was used as a template for site-directed mutagenesis. TAG stop codons were created at 4 different positions as indicated in fig.1, and the mutated inserts recloned into pEX34.

After transformation of *E. coli* strain Pop 2136, the fusion proteins produced upon induction were analyzed by Western blotting (fig.2). As the Pop strain is a C600 derivative of supF genotype, all transformants produced some wild-type sized SCY-MS2 fusion protein resulting from read-through in addition to the truncated version. In immunoblots all St-mutants were recognized by a polyclonal synaptophysin antiserum, while the mAb SY38 bound only to St1-MS2 and St2-MS2 fusion proteins but not to the shorter versions St3-MS2 and St4-MS2. The wild-type size proteins expressed from these DNAs have only a single amino acid exchange due to supF action. Indeed, they were all recognized by the mAb except for St3-MS2. Thus, the tyrosine residue at position 269 is located within the SY38 epitope (fig.2).

Four other independently isolated mAbs against synaptophysin, c7.1–c7.4, were also tested; the same result was obtained with all these antibodies (illustrated for mAb c7.4 in fig.3). Thus, the region between Tyr 269 and Tyr 289 defines a major immunogenic epitope of synaptophysin.

The repeat structure of SCY contains ten copies of an imperfect pentapeptide repeat (YGP(Q)QG; see fig.1). The most degenerate repeat, i.e. the region between Ser 278 and Tyr 283, coincides with the monoclonal an-

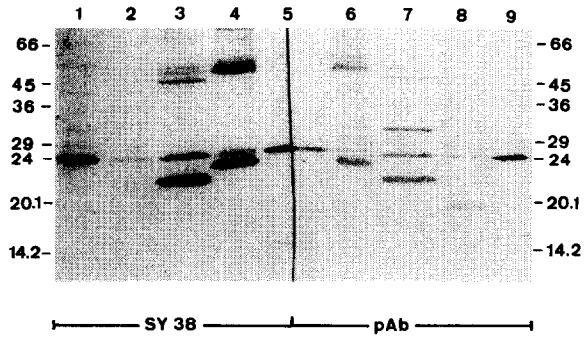

Fig.2. Western blot analysis of fusion proteins. Fusion proteins were expressed in *E. coli* after transfection with the various pEX34 constructs, electrophoresed on 10% SDS-PAGE and analyzed by Western blotting using either mAb SY38 (left) or a polyclonal antibody against synaptophysin (right). (Lanes 1 and 9) St4-MS2 construct; (lanes 2 and 8) St3-MS2 construct; (lanes 3 and 7) St2-MS2 construct; (lanes 4 and 6) St1-MS2 construct; (lanes 5 – both sides) SCY-MS2 fusion protein.

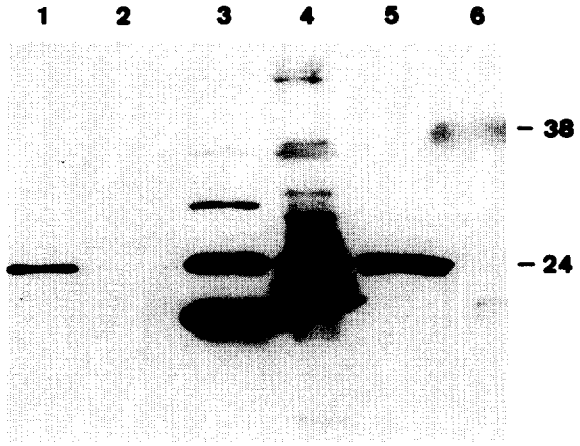

Fig.3. Western blot analysis of fusion proteins using mAb c7.4. For experimental details, see legend to fig.2. The following protein samples were applied: (lane 1) St4-MS2 construct; (lane 2) St3-MS2 construct; (lane 3) St2-MS2 construct; (lane 4) St1-MS2 construct; (lane 5) SCY-MS2 fusion protein; (lane 6) purified synaptophysin (500 ng). An identical picture was also obtained with mAbs c7.1, c7.2 and c7.3 (not shown).

tibody epitope. Flexible segments are considered highly immunogenic regions of proteins. Interestingly, by computer analysis the sequence (Pro-Ala-Ser-Gly-Gly-Gly-Gly) is predicted to belong to the most flexible regions in synaptophysin. We therefore believe that the repeat domain of the cytoplasmic tail forms a rigid structure interrupted by a highly immunogenic flexible segment, which represents a major portion of the SY38 epitope. In addition, Tyr 269 must be important as its replacement destroyed antibody binding.

Torpedo synaptophysin [13] exhibits 62% amino acid identity to the rat protein; its cytoplasmic region, however, is highly diverged and not recognized by antibodies specific for the epitope defined here [13]. Sequence comparison indeed reveals marked sequence differences in this region:

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          st3                          st2
         (269)                        (289)
           ||                           ||
Torpedo: YQPNYGQQEYPPQGG----DYQ
         || ||  || ||          ||      || ||
Rat:     YQPDYGQPASGGGGYGPQGDYG
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Thus, the antigenic domain of the mammalian protein most likely is not crucial to the channel function of this transmembrane protein.

The localization of the SY38 epitope presented here may be helpful for elucidating the effects of this antibody in functional assays. Interestingly, addition of SY38 mAb to synaptophysin reconstituted into planar lipid bilayer alters its channel properties [7]. Thus, the repeat structure of SCY may be implicated in conformational changes accompanying channel activation.

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