

Development of a New Epitope Tag Recognized by a Monoclonal Antibody to *Rickettsia typhi*

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ABSTRACT

The epitope recognized by a mouse monoclonal antibody (MAb) to the crystalline surface layer protein of *Rickettsia typhi*, SRT10, was mapped to 10 amino acid residues (SRTag TFIGAIATDT). The oligonucleotide sequence covering the epitope recognized by SRT10 was inserted into a mammalian expression vector together with multiple cloning sites. When the SRTag was fused in frame to the coding region of the NCC27/CLIC1 gene and expressed in mammalian cells, the MAb SRT10 could detect the tagged protein by immunoblotting, immunocytochemistry, and immunoprecipitation. In addition to the SRT-NCC27/CLIC1, SRT10 could detect N-terminal-tagged MEF2D and C-terminal-tagged CD4 by immunocytochemistry. We suggest that this specific recognition of the SRTag by SRT10 is generally applicable to cellular and molecular biology research that requires the expression and detection of fusion proteins.

INTRODUCTION

Epitope tagging and the antibody to the epitope are widely used in cellular and molecular biology research. When no antibodies to a particular protein are available, the construction of a fusion gene containing a particular epitope or Tag and the subsequent detection of its products by the anti-Tag antibody are a valuable alternative for the characterization of that protein. For example,

epitope tagging has been applied to elucidate intracellular location, post-translational modification, affinity purification, and interactions with other proteins of the tagged protein (2,7). Furthermore, to simultaneously express several ectopic genes and distinguish the gene products from endogenous proteins, several different Tags and antibodies that are sensitive and specific to those Tags are required.

Previously, we reported mouse monoclonal antibody (MAb) to the crystalline surface layer protein (SLP) of *Rickettsia typhi* and cloned the gene (*slpT*) encoding this protein (3). In this study, we determined the epitope that was recognized by one of our MAbs (SRT10 and IgG2a) to 10 amino acid residues of SLP. By tagging this epitope to a putative chloride channel protein, NCC27/CLIC1 (5,6), CD4 (4), and MEF2D (1), we examined the usefulness of this epitope tag and SRT10 as tools for molecular and cellular biology research.

MATERIALS AND METHODS

Epitope Mapping and Construction of Expression Plasmids

A series of deletion constructs of the *slpT* gene of *R. typhi* were prepared in pGEX™-4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden). By examining the reactivity of the SRT10 to bacterial lysates transformed with the expression constructs, we determined the epitope that was recognized by this MAb. On determining the epitope, its DNA sequence was inserted into the *SalI/EcoRI* sites of pCMV6 together with several cloning sites with oligonucleotides (Figure 1). Into this modified pCMV6 (pCMV6-SRT), we inserted the PCR-amplified cDNA of NCC27/

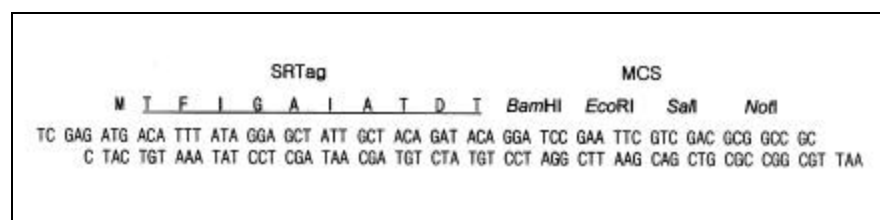


Figure 1. Construction of pCMV6-SRT plasmid. Oligonucleotides containing the coding region of SRTag and multiple cloning sites were inserted into pCMV6.

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CLIC1 (5,6). Oligonucleotides used for PCR were 5'-GACGGATCCATGGCTGAAGAACAAC-3' and 5'-TCCTCGAGGGGCTTATTTGAGGGC-3'. The underlining indicates the *Bam*HI and *Xho*I restriction sites, respectively. The resulting PCR product was cloned into the *Bam*HI/*Sal*I sites of the pGEX-4T-1 and the pCMV6-SRT for bacterial and mammalian cell expressions, respectively. CD4-SRT was created using the primers 5'-AAGTCTAGAAATGAA CCGGGAGTCCCTTTAG-3' and 5'-CTGGGATCCTCATGTATCTGTA GCAATAGTCCCTATAAAATGTAA TGGGGCTACATGTCTTCTGAAA-3'. SRT-MEF2D was created using primers 5'-CCGGAATTCATGGGG AGGAAAAAGATCAGATCCAGC-3' and 5'-TGGGTCGACTCACTTTAATGTCCAGGTATCAAGCCGCATCC-3'. The resulting PCR products of CD4-SRT and SRT-MEF2D were cloned into the *Xba*I/*Bam*HI sites of the pCMV6 and the *Eco*RI/*Sal*I sites of the pCMV6-SRT, respectively. Human thymus cDNA (Clontech Laboratories, Palo Alto, CA, USA) was used as a template for the reactions. The authenticity of the constructs was confirmed by automatic nucleotide sequencing.

Preparation of Rabbit Polysera to the NCC27/CLIC1

Recombinant GST-NCC27/CLIC1-fusion protein was prepared from *E.*

coli. Purified GST-NCC27/CLIC1 was cleaved with biotinylated thrombin (Novagen, Madison, WI, USA). Cleaved GST and thrombin were removed by Glutathione Sepharose® 4 B

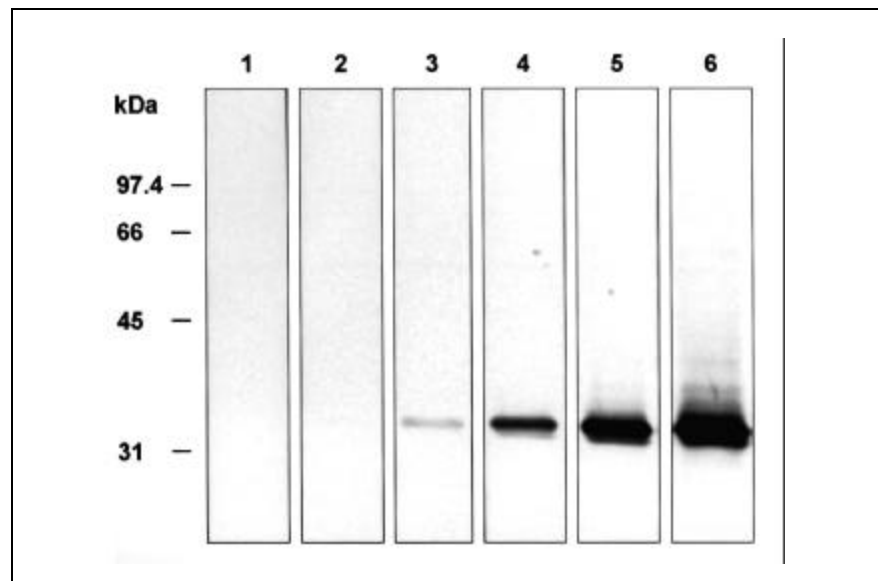


Figure 2. SRT10 recognizes the tagged NCC27/CLIC1 by immunoblotting. Untransfected cell lysate was probed with 1 µg/mL SRT10 (lane 1). SRT-NCC27/CLIC1-transfected cell lysates were probed with 0.1 (lane 2), 1 (lane 3), 10 (lane 4), 100 (lane 5), or 1000 ng/mL (lane 6) SRT10. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody was used as a secondary antibody. Molecular size standards are indicated on the left in kilodaltons.

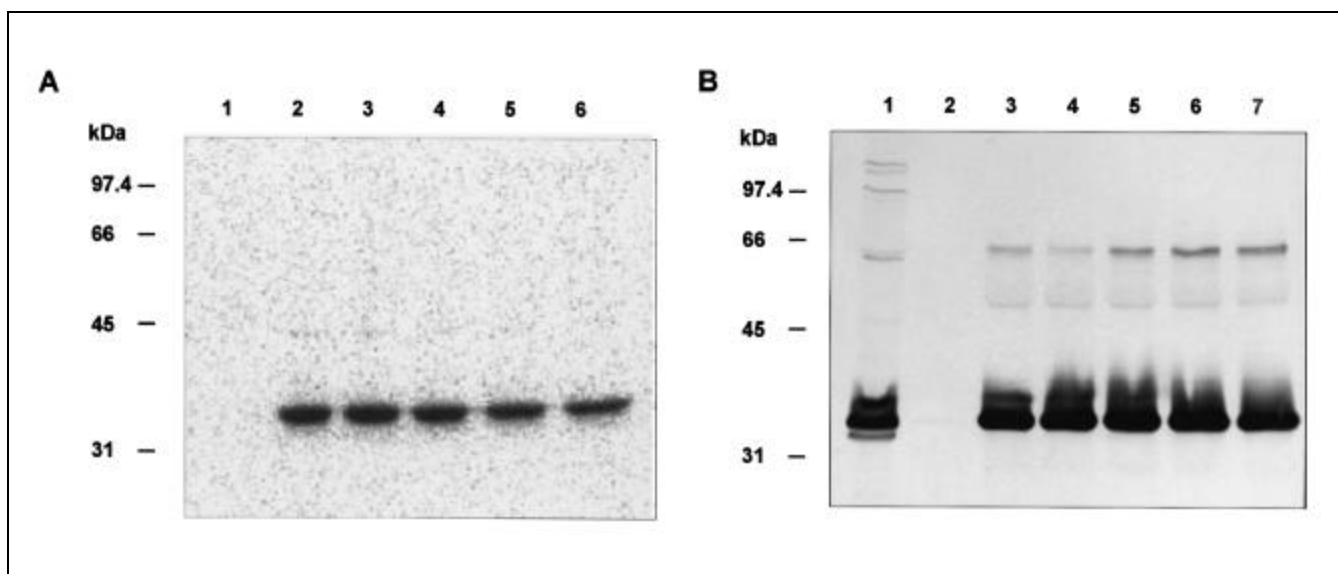


Figure 3. SRT10 precipitates the tagged NCC27/CLIC1 from mammalian cell lysates. (A) Radiolabeled lysates of SRT-NCC27/CLIC1-transfected cells were precipitated and subjected to autoradiography as described in Materials and Methods. Cell lysates (300 µg) were precipitated with a control antibody (lane 1) or SRT10 (lanes 2–6). Precipitated tagged protein and antibody complexes were washed with washing buffer containing 0.25 M (lanes 1 and 2), 0.5 M (lane 3), 0.75 M (lane 4), 1 M (lane 5), or 1.5 M (lane 6) NaCl. After SDS-PAGE, the gel was exposed to radiographic film for 50 h. (B) Lysates of SRT-NCC27/CLIC1-transfected cells were precipitated and subjected to immunoblotting. In lane 1, 75 µg total cell lysate (50% of input) was loaded. Cell lysates (150 µg) were precipitated with control antibody (lane 2) or SRT10 (lanes 3–7). Precipitated tagged protein and antibody complexes were washed with washing buffer containing 0.25 M (lanes 2 and 3), 0.5 M (lane 4), 0.75 M (lane 5), 1 M (lane 6), or 1.5 M (lane 7) NaCl. After SDS-PAGE and transfer, the blotted membrane was probed by rabbit anti-NCC27/CLIC1 antibody. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody was used as a secondary antibody.

(Amersham Pharmacia Biotech) and streptavidin agarose, respectively. The resulting cleaved and purified NCC27/CLIC1 was used as an immunogen injected into rabbits. Three injections were performed every two weeks. Two weeks after the last immunization, sera were collected.

Cell Culture and Transfection

Human embryonic kidney (HEK 293), HeLa, and C₂C₁₂ cells were maintained in DMEM supplemented with 10% FCS. HEK293 cells were transiently transfected with the expression plasmid by a calcium phosphate precipitation method. Cells were incubated for 8 h with the transfection solution, washed with PBS, returned to culture with fresh media, and grown for another 24 h. HeLa and C₂C₁₂ cells were transiently transfected by LIPOFECTAMINE™ (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. For radiolabeling, transfected HEK293 cells were incubated for 3 h with 100 µCi/mL L-[³⁵S]methionine and 100 µCi/mL L-[³⁵S]cysteine in methionine- and cysteine-deficient medium (Life Technologies).

Immunoblot Analysis

Transfected HEK293 cells were lysed in 50 mM Tris, pH 8.0, and 0.5% Nonidet® P-40 containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM aprotinin, and 2 mM leupeptin). Lysates were cleared by centrifugation at 15 000× g for 10 min. The protein contents of the resulting supernatants were determined by BCA™ kit (Pierce Chemical, Rockford, IL, USA). Proteins (20 µg/lane) were separated by SDS-PAGE using 10% polyacrylamide gels, transferred to nitrocellulose membrane, and probed with the MAb SRT10 (0.1, 1, 10, 100, or 1000 ng/mL). Untransfected cell lysate used as a control was probed with SRT10 at 1 µg/mL. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody (16 ng/mL) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the secondary antibody, followed by colorimetric detection with nitroblue tetrazolium and bromochloroindolyl phosphate.

Immunoprecipitation

The preparation of transfected cell lysates was performed with the methods previously described. Immunoprecipitation was performed by adding 8 µg SRT10 to 300 µg radiolabeled cell lysates and incubating for 1 h at 4°C with constant rotation. Protein A-Sepharose® beads (Amersham Pharmacia Biotech) were then added and incubated in the same condition for another hour. Immune complexes were then centrifuged for 2 min at 4000× g, washed twice in lysis buffer and twice in washing buffer (lysis buffer with 0.25, 0.5, 0.75, 1, or 1.5 M NaCl), and resuspended in SDS gel-loading buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Another MAb to the SLP of *R. typhi* (IgG2a) was also used as a control for the precipitation and washed in washing buffer with 0.25 M NaCl. Samples were then analyzed by SDS-PAGE and autoradiography. The gel was exposed to radiographic film for 50 h.

In a separate experiment, the precipitates were analyzed by immunoblotting. In this case, 150 µg cell lysates were precipitated with 4 µg SRT10. After SDS-PAGE and transfer, the blotted membrane was probed by the anti-NCC27/CLIC1 antibody previously described. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (0.4 µg/mL) was used to detect antibody-binding sites.

Immunocytochemistry

HeLa or C₂C₁₂ cells grown on microscope cover glasses and transfected with the expression plasmid SRT-NCC27/CLIC1, CD4-SRT, or SRT-MEF2D were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton® X-100. After washing them in PBS, the cells were incubated in 3 µg/mL SRT10, washed three times with PBS, incubated in 30 µg/mL FITC-conjugated goat anti-mouse IgG antibody (ICN Biochemicals, Costa Mesa, CA, USA), washed three times with PBS, and mounted with Fluoro-Guard™ (Bio-Rad Laboratories, Hercules, CA, USA). The prepared cells were examined by laser scanning mi-

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croscopy (LSM 510; Carl Zeiss, Jena, Germany).

RESULTS AND DISCUSSION

Epitope Mapping

By the analysis of a series of deletion constructs of the *slpT* gene of *R. typhi*, we determined the epitope that was recognized by the MAb SRT10 (3) as the 10 amino acid residues, TFIGA IATDT (SRTag).

Immunoblot Analysis

To test whether inserting the SRTag into a different sequence environment would affect its antigenicity, this epitope was fused in frame to the N-terminus of NCC27/CLIC1. This plasmid construct was transfected, and the SRT-NCC27/CLIC1 was expressed in HEK293 cells. After measuring the protein content, we subjected 20 μ g total cell lysate to immunoblotting. As shown in Figure 2, SRT10 recognized the SRT-NCC27/CLIC1—even at 1 ng/mL antibody concentration. Detectable cross-reactivity with the HEK293 proteins was not observed. When the blot was probed with the secondary antibody (80 ng/mL goat anti-mouse IgG antibody), this secondary antibody cross-reacted with several cellular proteins. However, at this concentration, signals from the tagged proteins were recognized at 0.1 ng/mL SRT10 (data not shown). When we expressed the deletion constructs of SLP in *E. coli* and examined their products with immunoblotting, no detectable cross-reactivity with *E. coli* proteins was observed (data not shown). Thus, this MAb can recognize the denatured tagged protein sensitively and specifically by immunoblotting.

Immunoprecipitation

To test whether SRT10 could precipitate tagged protein from mammalian cell lysate, transfected 293HEK cells were subjected to immunoprecipitation (Figure 3). Using autoradiography, we observed no detectable signal except SRT-NCC27/CLIC1, which suggested a specific binding of SRT10 (Figure

3A). When the concentrations of NaCl in washing buffers were increased from 0.25 to 1.5 M, the amount of precipitated SRT-NCC27/CLIC1 did not decrease, suggesting a high-affinity binding of the SRT10 to the SRT-tagged NCC27/CLIC1. By immunoblotting, we also confirmed that the precipitated protein is the NCC27/CLIC1 (Figure 3B). When the cell lysate was precipitated with the control antibody described in Materials and Methods, precipitation of the tagged protein was not observed (Figure 3A, lane 1, and Figure 3B, lane 2). Compared with the signal

by the SRT-NCC27/CLIC1 from the total cell lysate (Figure 3B, lane 1), about 70% of the tagged proteins were precipitated by 4 μ g SRT10 in this condition. Thus, this antibody can precipitate the tagged protein from mammalian cell lysate efficiently.

Immunocytochemistry

To test whether SRT10 could detect intracellular tagged protein, HeLa or C₂C₁₂ cells were transfected with SRT-tagged NCC27/CLIC1, CD4, or MEF2D, subjected to immunocyto-

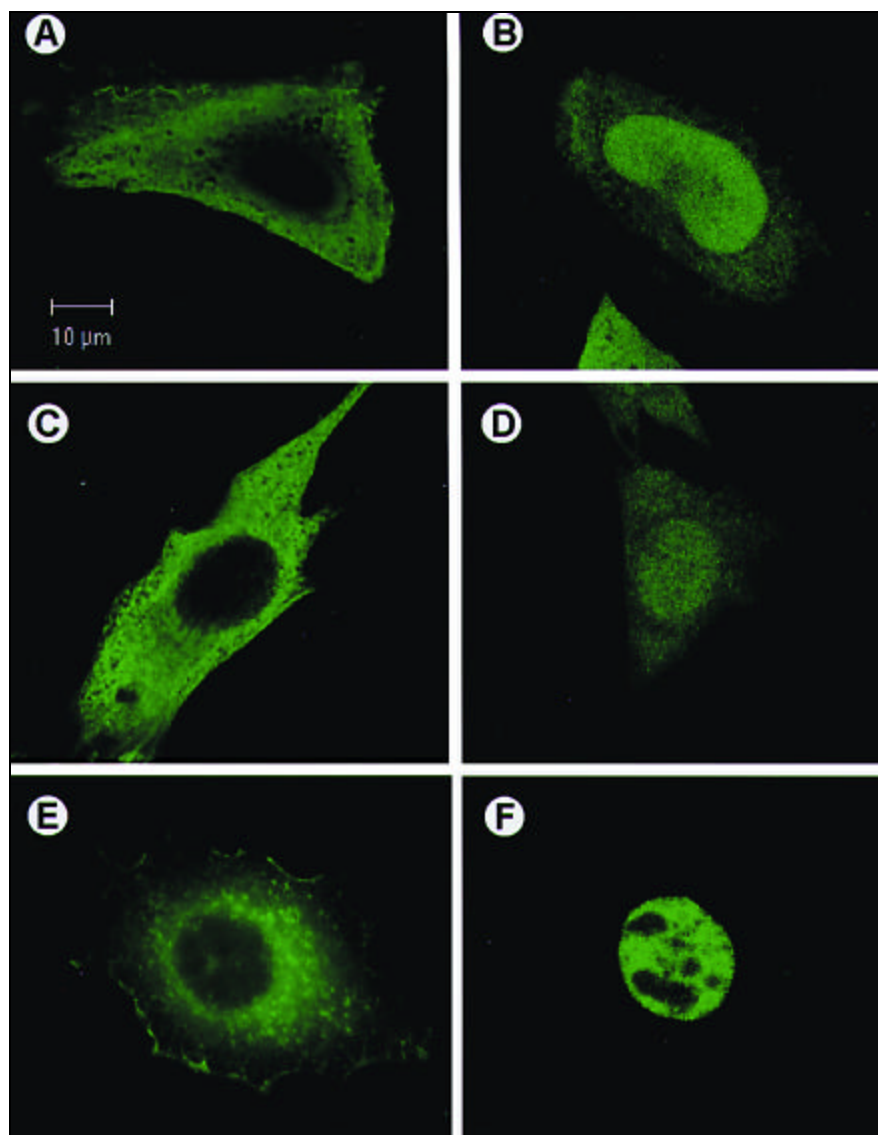


Figure 4. SRT10 recognizes intracellular SRT-NCC27/CLIC1. HeLa (A and B) and C₂C₁₂ (C and D) cells were transfected with the expression plasmid SRT-NCC27/CLIC1. Constructs of SRT-CD4 (E) and MEF2D (F) were transfected to HeLa cells. Transfected cells were stained with SRT10 and FITC-conjugated goat anti-mouse IgG antibody. Prepared samples were examined by confocal microscopy.

chemistry, and examined by confocal microscopy (Figure 4). Most of the NCC27/CLIC1-transfected C₂C₁₂ cells were stained prominently in the cytoplasm; however, a few cells were stained prominently in the nucleus. In NCC27/CLIC1-transfected HeLa cells, the antibody stained dominantly in the nucleus; however, some cells were stained dominantly in the cytoplasm. It is likely that the localization of the NCC27/CLIC1 may change between the nucleus and cytoplasm during the cell cycle. HeLa cells transfected with CD4-SRT were stained in the cytoplasmic membrane and cytoplasm. In SRT-MEF2D-transfected HeLa cells, the antibody stained dominantly in the nucleus. In adjacent, nontransfected cells, no detectable staining was observed (data not shown). Thus, this MAb can detect the tagged proteins by immunocytochemistry.

In this report, we determined the linear epitope that was recognized by the mouse MAb SRT10 to the rickettsial protein, SLP. By tagging this epitope to the N-terminus of NCC27/CLIC1, we showed that SRT10 could detect this tagged protein by immunoblotting, immunoprecipitation, or immunocytochemistry. We also showed that this antibody could detect N-terminal-tagged MEF2D and C-terminal-tagged CD4 by immunocytochemistry. Because this MAb is both sensitive and specific, this tag and its MAb should be generally applicable to cellular and molecular biology research. Interested individuals can obtain antibody SRT10 from the authors.

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