Identification of Novel Bifunctional Calmodulin-binding and Microtubule-stabilizing Motifs in STOP Proteins*

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Christophe Bosc‡‡, Ronald Frank§, Eric Denarier‡‡, Michel Ronjat‡, Annie Schweitzer‡, Jürgen Wehland‡‡, and Didier Job‡‡

From the ‡Commissariat à l’Énergie Atomique-Laboratoire du Cytosquelette, INSERM Unité 366, Département de Biologie Moléculaire et Structurale/Cytosquelette, Commissariat à l’Énergie Atomique-Grenoble, F-38054 Grenoble cedex 9, France, §AG Molecular Recognition, GBF, Mascheroder Weg 1, D-38124, Braunschweig, Germany, ‡Commissariat à l’Énergie Atomique-Laboratoire Canaux Ioniques et Signalisation, INSERM U9931, Département de Biologie Moléculaire et Structurale/Canaux Ioniques et Signalisation, Commissariat à l’Énergie Atomique-Grenoble, F-38054 Grenoble cedex 9, France, and the ‡‡Department of Cell Biology, Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-38124, Braunschweig, Germany

Although microtubules are intrinsically labile tubulin assemblies, many cell types contain stable polymers, resisting depolymerizing conditions such as exposure to the cold or the drug nocodazole. This microtubule stabilization is largely due to polymer association with STOP proteins. There are several STOP variants, some with capacity to induce microtubule resistance to both the cold and nocodazole, others with microtubule cold stabilizing activity only. These microtubule-stabilizing effects of STOP proteins are inhibited by calmodulin and we now demonstrate that they are determined by two distinct kinds of repeated modular sequences (Mn and Mc), both containing a calmodulin-binding peptide, but displaying different microtubule stabilizing activities. Mn modules induce microtubule resistance to both the cold and nocodazole when expressed in cells. Mc modules, which correspond to the STOP central repeats, have microtubule cold stabilizing activity only. Mouse neuronal STOPs, which induce both cold and drug resistance in cellular microtubules, contain three Mn modules and four Mc modules. Compared with neuronal STOPs, the non-neuronal F-STOP lacks multiple Mn modules and this corresponds with an inability to induce nocodazole resistance. STOP modules represent novel bifunctional calmodulin-binding and microtubule-stabilizing sequences that may be essential for the generation of the different patterns of microtubule stabilization observed in cells.

Microtubules are key cytoskeletal elements in eukaryotic cells and are involved in many vital processes such as cell morphogenesis and division (1). Pure tubulin microtubules have intrinsic dynamic properties (2, 3) and are labile structures, rapidly depolymerized upon exposure to the cold or to the drug nocodazole (1). In cells, microtubule dynamics and stability properties are tightly controlled (4–6). Many cell types contain stable microtubules which resist depolymerizing conditions such as exposure to the cold or nocodazole (7–10). Cold stability and nocodazole resistance represent distinct properties of microtubules. In neuronal cells, axonal microtubules are both cold- and drug-resistant (11, 12) whereas in fibroblastic cells, many cytoplasmic microtubules are cold-stable but drug-labile (10, 13). Recently, the involvement of the calmodulin-binding and microtubule-associated proteins STOP in these aspects of microtubule stabilization has become evident. STOP proteins were initially characterized as microtubule cold-stabilizing factors whose activity was inhibited by interaction with Ca$^{2+}$-calmodulin (14–17). There are several STOP variants whose expression varies among tissues and during development. Neurons contain two major variants of STOP, N-STOP and E-STOP (18, 12). In mice, fibroblasts express one major STOP variant, F-STOP (13). When expressed in cells, the neuronal N- or E-STOP associate with cytoplasmic microtubules at physiological temperature and induce microtubule resistance to both the cold and nocodazole (18, 12). In contrast, in similar conditions, the fibroblastic F-STOP associates with interphase polymers only during cold exposure, being almost entirely diffusely distributed throughout the cytoplasm in interphase cells at physiological temperature (13). Consequently, F-STOP induces extensive microtubule cold stability without any detectable effects on microtubule behavior at physiological temperature, including microtubule sensitivity to nocodazole. Injection of STOP neutralizing antibodies suppresses the microtubule resistance to both the cold and nocodazole normally observed in neuronal cells (12), and the cold stability of cytoplasmic microtubules in fibroblastic cells (13). Thus, STOP proteins are key factors responsible for the different patterns of microtubule stabilization observed in cells.

Although the behavior and stabilizing activity of STOP proteins in cells are now firmly established, very little is known concerning the STOP domain structure. In particular, information concerning the number and location of calmodulin- and microtubule-binding sites on the proteins has been lacking. Consequently, the molecular basis for the different activities of the various STOP variants and the mechanism of STOP regulation by calmodulin are unknown. In this study we have combined new methods of detection of protein-protein interactions (immobilized peptide arrays) with biochemical and cellular assays of STOP activity to probe the STOP domain

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‡ To whom correspondence should be addressed: CEA-Laboratoire du Cytosquelette, INSERM Unité 366, DBMICS, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble cedex 9, France. Tel.: 33-476885955; Fax: 33-476885057; E-mail: cbosc@cea.fr.

1 The abbreviations used are: N-STOP, neuronal adult STOP; aa, amino acid(s); CaM-D, dapsylated calmodulin; E-STOP, early-STOP; F-STOP, fibroblastic STOP; CaM, calmodulin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.
structure. We demonstrate that STOP has multiple calmodulin-binding sites which partially overlap with two distinct sets of microtubule-stabilizing motifs. The motif composition of STOP variants determines the type of microtubule stabilization which these variants generate in cells. Furthermore, the presence of STOP motifs in apparently unrelated proteins indicates that they may be functional components shared by several diverse cell effectors.

**EXPERIMENTAL PROCEDURES**

**Construction of STOP cDNA Deletion Mutants—cDNAs coding for a series of STOP mutants were constructed, using standard molecular biology techniques. Using as numbering as in rat N-STOP protein (18), these mutants were: Nt (aa 1–174), LNT (aa 1–225), SNT (Met + aa 90–174), 3R (Met + aa 221–455), 4R (Met + aa 221–267 + aa 314–455), 3R (Met + aa 313–455), 2R (Met + aa 359–455), 1R (Met + aa 405–455), and LCT (Met + aa 451–952). Δ1, Δ2, Δ3, and Δ5 correspond to the deletion of calmodulin-binding peptides Cam1, Cam2, Cam3, and Cam5 as described in the text. Δ1 results to the deletion of aa 2–19. Δ2 results to the replacement of aa 124–138 by Ala. Δ3 results to the deletion of aa 162–171 or to the replacement of aa 162–174 by Ile-Glu, in mutants derived from LNT and from other STOP fragments, respectively. Δ5 results in the deletion of aa 481–495. The constructs were derived from plasmids pSG5–STOP (18) and p16CaApo (13). All cDNAs were cloned in vector pSG5 (Stratagene, La Jolla, CA), with a Kozak consensus (19).

**In Vitro Translation of Calmodulin, N-STOP, and STOP Mutants—cDNAs coding for calmodulin, N-STOP, and the STOP mutants described above were in vitro translated in the presence of 35S-labeled calmodulin, mixed with increasing concentrations of F-STOP (0.5 to 3.1 μM) was added stepwise to increasing concentrations (total added volume of 30 μL). For Scatchard representation, the bound calmodulin concentration was calculated at each calmodulin concentration as [ΔF/ΔF_{max}] × [total calmodulin]. The free F-STOP concentration was calculated as (total calmodulin) – [bound calmodulin].**

**Protein Concentrations—**For F-STOP binding, protein concentration was determined using both the Micro-BCA Protein Assay Reagent Kit from Pierce (Rockford, IL) and the Bio-Rad Protein Assay (Bio-Rad), using bovine serum albumin as standard. Results were similar for both assays. The concentration of calmodulin was determined using ultraviolet spectroscopy and an extinction coefficient of ε_{280} = 3300 m^–1 cm^–1.

**Transient Transfection and Immunofluorescence Analysis of Microtubule Stability—**Exponentially growing HeLa cells were transfected with cDNAs coding for either N-STOP or the STOP mutants described above, using FuGENE 6 (Roche Diagnostics, Meylan, France) as a transfection reagent, according to the manufacturers instructions. For control of transfection, efficiency cells were co-transfected with plasmid pEGFP-C2 (CLONTECH, Palo Alto, CA). Transfected cells were either further exposed to cold temperature (30 min on ice) or to nocodazole (20 μM for 30 min). Cells were then permeabilized in lysis buffer (80 μM Pipes, 1 mM EGTA, 1 mM MgCl2, 10 μM Triton X-100, pH 6.75) and fixed for 6 min at −20 °C in methanol. Microtubules were stained with β-tubulin mAb TUB2.1 (1:100, Sigma) and anti-mouse Cy3-coupled secondary antibody (I:1000, Jackson Immunoresearch Laboratories, West Grove, PA). Images were digitalized using a Princeton RTE-CCD–1317K/1 camera (Princeton Instruments, Trenton, NJ) and IPLab Spectrum software (Signal Analytics, Vienna, VA).

**STOP Sequences and Data Base Searches of STOP Analogs—**Accession numbers for known STOP sequences are: CA63762 for rat N-STOP, JC5963 for mouse N-STOP,CAA75989 for mouse F-STOP, and AAC79958 for chicken E-STOP (cNau). Mouse E-STOP protein sequence was deduced from mouse genomic sequences (GenBank™ accession numbers Y14754 and Y14755). Searches for novel STOP proteins or analogs were performed using the BLAST program available at www.ncbi.nlm.nih.gov/BLAST/. The human N-STOP protein sequence was deduced from human genomic sequence (GenBank™ accession number AP001922). Fugu STOP protein sequence was deduced from the Fugu GenBank™ genomic sequences (accession numbers AL018884, AL019006, AL019013, AL019018, and AL020541) and from the UK HGMP-RC Fugu Project (fugu.hgmp.mrc.ac.uk) genomic sequence 169C21bAI. Tetraodon STOP protein sequence was deduced from Tetraodon genomic sequences (GenBank™ accession numbers AL233485, AL250750, AL253833, AL259813, and AL299298).
STOP proteins have two salient properties: they behave as *bona fide* calmodulin-binding proteins *in vitro*, being absorbed on Ca$^{2+}$-calmodulin columns at high ionic strength; they have the capacity to induce microtubule stabilization in cells. To localize the STOP sequences responsible for these biochemical and functional properties of the protein, we assayed a series of STOP deletion mutants for capacity to bind to immobilized calmodulin and to stabilize cellular microtubules. As a preliminary step to facilitate the search for STOP sequences interacting with calmodulin, we used immobilized peptide arrays to detect candidate calmodulin-binding peptides in N-STOP.

**Identification of Calmodulin-binding Peptides in N-STOP**—Immobilized peptide arrays have been initially used for epitope mapping in studies of antibody-antigen interactions (23). Subsequently, immobilized peptides have been used successfully for the mapping of the binding domains involved in other types of protein-protein interactions (24–26). A basic condition for successful use of the technique is that the interaction under study involves linear peptide stretches on at least one of the partners. This seemed to be the case for calmodulin interaction with target proteins. Known calmodulin-binding sites on proteins comprise linear aa stretches of about 15–30 aa in length, with a core domain of about 14 aa (27). As a preliminary test of the method, we assayed calmodulin binding to immobilized peptides corresponding to the calmodulin-binding site of CaM-kinase II (28) and its flanking sequences. A single cluster of positive spots was detected, corresponding to the known calmodulin-binding domain of CaM-kinase II (Fig. 1A). Applied to N-STOP which is the largest variant of STOP and which contains all known STOP sequences, the same method yielded at least 12 clusters of overlapping peptides (Fig. 1B), corresponding to 12 peaks of radioactivity upon quantitative analysis (Fig. 1C). As a control for the Ca$^{2+}$ dependence of the calmodulin interaction with STOP peptides, an N-STOP peptide-membrane was incubated with $^{35}$S-labeled calmodulin in the presence of excess EGTA. No detectable binding of $^{35}$S-labeled calmodulin to STOP peptides was observed in this condition (not shown).

The position of the 12 $^{35}$S-radioactive peaks in the N-STOP sequence is shown in Fig. 1D and Table I shows the aa sequences of the 12 putative N-STOP calmodulin-binding peptides (corresponding to the maximum of the radioactivity peaks shown in Fig. 1C). N-STOP contains two distinct repeat domains, a central repeat domain containing 5 tandem repeats of 46 aa and a carboxyl-terminal repeat domain containing 28 repeats of an 11-aa motif (18). Four calmodulin-binding peptides were located upstream of the central repeats. Each central repeat contained a calmodulin-binding peptide. Three calmodulin-binding peptides were located between the central repeats and the carboxyl-terminal repeat domain. Only weak signals were observed in this latter STOP domain.

These results suggested the presence of multiple and specific Ca$^{2+}$-calmodulin-binding sites on N-STOP. However, this multiplicity of sites was not completely expected and we tested whether multiple calmodulin-binding sites were indeed accessible in STOPs. We used F-STOP for test because we have succeeded in producing this STOP variant using a baculovirus expression system. According to immobilized peptide analysis of N-STOP, F-STOP is predicted to contain six calmodulin-binding peptides (CamR1, CamR2, CamR4, CamR5, Cam5, and Cam6, Fig. 2A). Scatchard analysis of calmodulin binding to F-STOP showed a biphasic graph, compatible with the presence of three to four binding sites with a $K_d$ of 1.7 $\mu M$ and of additional binding sites with a $K_d$ of 8.1 $\mu M$ (Fig. 2B) on each F-STOP molecule. Within the scope of the present paper, the important finding was that the Scatchard plot yielded an estimate of a total of about 7 calmodulin-binding sites per F-STOP molecule, in reasonable agreement with the number of calmodulin-binding peptides detected using immobilized peptide analysis (six). As a control of our analysis and of the reliability of our binding site concentration estimates, we assayed F-STOP binding to calmodulin in conditions in which the probability of binding several calmodulin molecules on each F-STOP molecule was minimized (see methods, Fig. 2C). In such conditions, a linear Scatchard plot was observed and yielded a calculated binding site concentration of 175 nM in good agreement with the actual concentration of calmodulin in the assay, *i.e.* 200 nM (Fig. 2B).

![Fig. 1](image-url) Identification of calmodulin-binding peptides using immobilized peptide arrays. A, control experiment: $^{35}$S-labeled calmodulin overlay of a membrane containing immobilized peptides corresponding to the regulatory segment (aa 271–348) from human CaM-kinase II $\beta$ chain (autoradiography). The sequence of the peptide cluster interacting with $^{35}$S-labeled calmodulin is indicated. The calmodulin-binding site sequence described in Ref. 28 is underlined. B, $^{35}$S-labeled calmodulin were detected on autoradiography. C, quantitative analysis of $^{35}$S-calmodulin binding to STOP peptides. The radioactive signals observed in B were quantified and results were plotted. Peptides are numbered as in B and signal values are in arbitrary units. D, mapping of the radioactivity peaks observed in C, on a schematic representation of rat N-STOP. Five calmodulin-binding sites (R1-R5) were located in the central repeats. Other calmodulin-binding sites (1–7) are numbered from the amino terminus to the carboxyl terminus of the protein. Central Rpt, central repeat domain. C-terminal Rpt, carboxyl-terminal repeat domain.
TABLE I
Amino acid sequence of the STOP calmodulin-binding peptides

| Peptide | Sequence | Amino acid position |
|---------|----------|---------------------|
| Cam1    | MWPICITRACCIARF | 1–15 |
| Cam2    | RAWKVRPEPSRPR   | 124–138 |
| Cam3    | AWPLPRGDPHPWIPK | 160–174 |
| Cam4    | PVLGMKPRPGGQGER | 187–201 |
| CamR1   | TRRKAGPAWMYRTTE | 235–249 |
| CamR2   | DTRRKAGPAWMVTRT | 280–294 |
| CamR3   | RDRTRKAGPAWMYTR | 325–339 |
| CamR4   | TRRKAGPAWMYRTTE | 373–387 |
| CamR5   | RKAGPAWMVRSQEGH | 421–435 |
| Cam5    | RAWTDIKPMVIPK    | 481–495 |
| Cam6    | RRRRISLYSEPFKES | 532–546 |
| Cam7    | PPKSTSSQKPKLRK  | 559–573 |

Taken together, these results indicate the presence of multiple calmodulin-binding sequences in STOP proteins and yield an identification of candidate calmodulin-binding sequences in STOP. This information proved to be most useful for mutant analysis, as shown below.

**Assay of N-STOP Fragment and Deletion Mutants Binding to Immobilized Calmodulin—**N-STOP binds to immobilized calmodulin columns in the presence of 0.4 M NaCl and 1 mM free Ca\(^{2+}\) (18). We wished to identify the N-STOP sequences responsible for this solid interaction of the protein with immobilized calmodulin. To do this we tested STOP fragments and deletion mutants for capacity to bind to immobilized calmodulin at high ionic strength, in a Ca\(^{2+}\)-dependent manner. STOP fragments and mutants were produced by *in vitro* translation of the corresponding cDNAs in reticulocyte lysates containing \(^{35}S\)-labeled methionine. Fig. 3 shows typical chromatography profiles corresponding to a positive (A to C) and a negative result (D). In experiments scored as positive, translation products were retained on calmodulin affinity columns in the presence of Ca\(^{2+}\) and 0.4 M NaCl (Fig. 3A, compare lanes L and F), whereas the bulk of lyase proteins were eluted in the column flow-through (Fig. 3B). The bound translation products could be eluted from the column in the presence of EGTA (Fig. 3A, lanes 2–6). Additionally, the same translation products failed to bind to control columns run in the presence of EGTA (Fig. 3C). In experiments scored as negative, translation products failed to bind to immobilized calmodulin, even in the presence of Ca\(^{2+}\) (Fig. 3D). Thus, differences between positives and negatives were clear-cut.

**Localization of the Sequences Responsible for N-STOP Binding to Immobilized Calmodulin—**For mapping of the sequences responsible for N-STOP binding to immobilized calmodulin, we first applied the column assay described above to large protein subdomains, then to subdomains deleted for the putative calmodulin-binding peptides identified on immobilized peptide arrays. The protein was subdivided into three subdomains corresponding to the amino-terminal domain located upstream of the central repeats (LNt), the central repeats (5R), and the carboxyl-terminal domain located downstream of the central repeats (LCt), respectively (Fig. 4A). The STOP domains containing either the amino-terminal domain of the protein (LNt) or the central repeats (5R) bound to immobilized calmodulin columns. In contrast, the carboxyl-terminal region of the protein (LCt) did not bind to immobilized calmodulin. Subdomains LNt and 5R were subjected to further deletion mutant analysis. A mutant (LNtΔ1Δ2Δ3), containing peptide Cam4 but deleted for peptides Cam1, Cam2, and Cam3, did not bind to immobilized calmodulin (Fig. 4B). This result indicated that peptide Cam4 may not be significantly involved in LNt interaction with immobilized calmodulin. A mutant (Nt) restricted to the LNt sequences containing peptides Cam1, Cam2, and Cam3 interacted with immobilized calmodulin. In contrast, a mutant of Nt (NtΔ1Δ2Δ3) deleted for peptides Cam1, Cam2, and Cam3 was devoid of calmodulin-binding capacity. Furthermore, deletion

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**Fig. 2. Fluorescence analysis of the interaction of dansyl-calmodulin (CaM-D) with F-STOP.** A, schematic representation of the mouse F-STOP (13) showing the presence of calmodulin-binding peptides CamR1, CamR2, CamR4, CamR5, Cam5, and Cam6. Calmodulin-binding sites are denoted as in Fig. 1 and also indicated by an asterisk. B, Scatchard analysis of calmodulin binding to F-STOP. F-STOP (600 nM) was incubated with CaM-D (400 nM) in the presence of increasing concentrations of non-dansylated calmodulin. The resulting Scatchard plot was biphasic. The *upper* and *lower* branches of the curve were fitted with regression lines calculated using the first seven points and the last seven points of the plot, respectively. **Kd** values and total binding site concentrations (determined by the intercepts of the regression lines with the *abscissa*) are indicated for both regressions. The corresponding total numbers of calmodulin-binding sites per F-STOP molecule, estimated from the ratios of the total binding site concentrations divided by the total F-STOP concentration (600 nM), are also indicated. Results indicate a total of about 7 calmodulin-binding sites per F-STOP molecule, including 3–4 sites with a **Kd** of 1.7 μM and additional sites with a **Kd** of 8.1 μM. C, Scatchard analysis of F-STOP binding to dansyl-calmodulin. This experiment was introduced as a control of the reliability of binding site concentration estimates. CaM-D (200 nM in MEM, 100 mM NaCl, 2 mM CaCl₂) was incubated with F-STOP at increasing concentrations (0.5 to 3.1 μM). The corresponding **Kd** and estimated binding site concentration are indicated. This latter concentration (178 nM) was in good agreement with the actual concentration of CaM-D (200 nM).
mutants of Nt containing only one of the peptides Cam1 (Nt\(\Delta1\Delta2\Delta3\)), Cam2 (Nt\(\Delta1\Delta3\)), or Cam3 (Nt\(\Delta1\Delta2\)) showed calmodulin-binding capacity. These results indicate that peptides Cam1, Cam2, and Cam3 are essential for N-STOP N-terminus binding to immobilized calmodulin and that each one of these peptides suffices to confer calmodulin-binding capacity to this STOP domain.

FIG. 3. Assay of STOP mutant binding to immobilized calmodulin. STOP mutants were produced using in vitro translation of STOP cDNA constructs, using reticulocyte lysates containing \(^{35}\text{S}\)methionine. Lysates were loaded into calmodulin-agarose columns in buffer in the presence of 1 mM Ca\(^{2+}\) (unless otherwise indicated) and 400 mM NaCl. Columns were washed with the same buffer and subsequently eluted with EGTA-containing buffer. Aliquots of the loaded sample (L), flow-through fraction (F), and EGTA elution fractions (1–6) were analyzed for translation product and protein content using SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining of the gels and autoradiography. A and B, results observed with a STOP mutant (5R) containing calmodulin binding activity. A, autoradiography showing binding of translation products to immobilized calmodulin. Translation products were absent from the flow-through fraction and eluted in the EGTA elution fractions. B, Coomassie Blue staining showing elution of the bulk of lysate proteins in the flow-through fractions. C, same as A in the presence of 2 mM EGTA in the loading and washing buffers. In the absence of free Ca\(^{2+}\), translation products did not bind to the column, being present in the flow-through fractions. D, results (autoradiography) observed with a STOP mutant (Nt\(\Delta1\Delta2\Delta3\)) devoid of calmodulin binding activity. Conditions were as in A. The mutant failed to bind to immobilized calmodulin in the presence of free Ca\(^{2+}\), being present in the column flow-through. Arrow, \(^{35}\text{S}\)-labeled translation product. Molecular masses are in kDa.

FIG. 4. Calmodulin-agarose binding of STOP fragments and deletion mutants. The calmodulin binding of individual STOP domains and STOP mutants was analyzed as shown in Fig. 2. + and – indicate binding and non-binding, respectively. A, calmodulin binding of N-STOP, of the amino-terminal domain of N-STOP (LNT) located upstream of the central repeats, of the N-STOP central repeat domain (5R), and the carboxyl-terminal domain of N-STOP (LCT) located downstream of the central repeats. B, analysis of the role of the calmodulin-binding peptides Cam1, Cam2, Cam3, and Cam4 in the calmodulin binding activity of fragment LNT. STOP fragments and deletion mutants containing various combinations of the calmodulin-binding peptides as indicated were tested for calmodulin binding activity. C, calmodulin binding of N-STOP central repeats. Calmodulin-binding sites are denoted as in Fig. 1 and also indicated by an asterisk. The central repeat and the carboxyl-terminal repeat domains of N-STOP are indicated as in Fig. 1.

The central repeat domain of STOP consists of five 46-aa tandem repeats, each containing a putative calmodulin-binding peptide (CamR1 to CamR5) in its center part (Fig. 1D). We designed a series of STOP fragments containing 5 to 1 repeats (5R to 1R) to determine the minimum number of repeats needed for significant interaction with immobilized calmodulin. We found that at least two central repeats were required...
for such an interaction (Fig. 4C), suggesting that individual repeats could act as cooperative individual binding units in the interaction of the STOP central repeat domain with immobilized calmodulin.

Our results strongly indicate that peptides Cam1, Cam2, and Cam3 present in the amino-terminal domain of the protein and peptides CamR1 to CamR5 present in the STOP central repeats, mediate STOP binding to immobilized calmodulin. They also indicate that no other STOP peptide is involved.

Patterns of Microtubule Stabilization Generated by N-STOP Domains—To localize the microtubule-stabilizing domains of N-STOP, we assayed the microtubule-stabilizing activity of a series of STOP subdomains and deletion mutants, using cellu-
lar assays. HeLa cells which are normally devoid of both cold-stable and drug-resistant microtubules (10) were transfected with the various STOP cDNAs. Transfected cells were examined for microtubule resistance to the cold and nocodazole. While transfection of HeLa cells with vector alone did not induce any microtubule stabilization (Fig. 5A and B), transfection with specific STOP cDNA fragments could induce microtubule resistance to nocodazole and/or microtubule cold stability. When a cDNA encoding the STOP central repeats (5R) was used for HeLa cell transfection, cytoplasmic microtubules showed extensive cold stability (Figs. 5C and 6A) but no significant degree of microtubule resistance to nocodazole (Figs. 5D and 6A). By contrast, cells transfected with cDNA corresponding to the amino-terminal region of N-STOP (LNt) showed resistance to both the cold (Figs. 5E and 6A) and nocodazole (Figs. 5F and 6A), and displayed the characteristic serpentine shape of nondynamic, detyrosinated microtubules (Refs. 29 and 30, and data not shown). As LNt, the carboxyl-terminal domain of N-STOP (LCt), induced microtubule resistance to both the cold and nocodazole when expressed in HeLa cells (data not shown and Fig. 6A).

These results indicate the presence of multiple microtubule-stabilizing sequences in N-STOP, distributed in all three amino-terminal, central and carboxyl-terminal subdomains of the protein. Furthermore, two distinct patterns of microtubule stabilization are observed. The N-STOP LNt and LCt subdomains induce microtubule resistance to both the cold and nocodazole, whereas the 5R subdomain induces microtubule cold stability only.

Mapping of the Microtubule Stabilizing Activity of N-STOP—The microtubule stabilizing activity of N-STOP is inhibited by calmodulin and this raised the possibility of overlap between calmodulin-binding sequences and microtubule-stabilizing sequences in the protein. Based on this possibility, we proceeded to analyze the microtubule stabilizing activity of N-STOP subdomains (LNt, 5R and LCt) deleted for the calmodulin-binding peptides identified on immobilized peptide arrays.

For analysis of the STOP amino-terminal domain, we designed a series of deletion mutants of LNt comprising various combination of peptides Cam1, Cam2, Cam3, and Cam4 (Fig. 6B). We found that a construct containing peptides Cam1 and Cam4 but devoid of peptides Cam2 and Cam3 (LNtΔ2Δ3) was completely inactive. In contrast, deletion mutants of LNt containing either peptide Cam2 (LNtΔ3) or peptide Cam3 (LNtΔ2) induced both microtubule cold stability and microtubule resistance to the cold when expressed in HeLa cells. Furthermore, a small fragment of the N-STOP amino terminus comprising peptides Cam2 and Cam3 (SNtΔ2Δ3) was fully active. The same fragment further deleted for peptides Cam2 and Cam3 (SNtΔ2Δ3) was completely inactive. These results indicated a close association between Cam2 and Cam3 sequences and the microtubule-stabilizing sequences of LNt. The SNt fragment of the N-STOP amino terminus was the smallest fully active fragment that we could derive from the N-STOP amino termi-
nus. Interestingly, this minimal microtubule-stabilizing fragment contained two homologous sequences of 15 aa (Fig. 7A, boxed aa). These two conserved peptides were located upstream of and partially overlapped calmodulin-binding peptides Cam2 and Cam3 (Fig. 7A, underlined aa), respectively. Thus, fragment SNt had a modular structure, containing two repeats of motifs covering a conserved sequence and an overlapping calmodulin-binding peptide. These motifs, will be referred to as Mn1 and Mn2 modules, respectively (Fig. 7A).

The carboxyl terminus of N-STOP contains three putative calmodulin-binding peptides as determined by peptide SPOT. These peptides were apparently not involved in the binding of N-STOP to immobilized calmodulin. Nevertheless, one of the peptides (peptide Cam5) was associated with the conserved sequence found in Mn1 and Mn2 modules, and is hence referred to as Mn3 (Fig. 7B). To test the possibility that the observed microtubule stabilizing activity of the STOP carboxyl terminus may involve Mn3 module, we assayed a construct deleted for peptide Cam5 (LCt/H9004). This mutant was totally devoid of microtubule stabilizing activity (Fig. 6C) indicating that the Mn3 module was indeed essential for the microtubule stabilizing activity of the N-STOP carboxyl terminus.

The combined five central sequence repeats of N-STOP can also confer cold stability to microtubules. We therefore assayed progressive sequence repeat truncation constructs in terms of microtubule cold stability. We found that at least two tandem repeats were needed for cold stabilization (Fig. 6D). This also corresponds to the minimum number of central repeats required for calmodulin binding activity (Fig. 4C). The simplest interpretation of these results is that individual N-STOP central repeats act cooperatively to confer both calmodulin binding activity and microtubule cold stabilizing activity to the central repeat domain of the protein. Analogous to the Mn modules, each individual STOP central repeat represents a second type of microtubule-stabilizing motif. They similarly comprise a calmodulin-binding site (CamR1 to CamR5) and are hence referred to as Mc modules.

Our results indicate that N-STOP contains two classes of microtubule-stabilizing modules. Mn modules, localized on both sides of the central repeat domain of N-STOP and Mc modules, constituting the N-STOP central repeat domain. STOP domains which contain Mn modules induce microtubule resistance to both the cold and nocodazole, while STOP central repeat domains (containing Mc modules), only induce microtubule cold stability. The two classes of modules show some homology, involving 5 aa (Fig. 7C). Further structural studies will tell us whether or not these aa are of particular importance for microtubule stabilization.

**Phylogenetic Variation of STOP Mn and Mc Motif Arrangement**—A search of sequence data bases has revealed STOP-related proteins in a number of animal species. Fig. 8 shows schematic representations of rat N-STOP, the three splicing variants of mouse STOP, and STOP proteins identified in other species. The structure of mouse and human N-STOP is similar to that of rat N-STOP and the overall sequence homologies with rat N-STOP are 94 and 79%, respectively. Variations between the three species are mainly restricted to the number of central repeats (five in the rat N-STOP, four in the mouse N-STOP, and only one in the human N-STOP). An avian E-STOP protein ortholog has been cloned and sequenced in
chicken (31) while partial fish STOP sequences (Fugu and Tetraodon) have also been reported (Fig. 8). Quite interestingly, the avian and fish proteins both lack the central repeat region present in mammalian STOP proteins. Upstream and downstream of this region, the homology with mammalian proteins is extensive. The homology between chicken, Fugu,
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and Tetraodon STOPs with the corresponding rat N-STOP sequences is 82, 71, and 74\%, respectively. Mn modules are conserved among all species. The divergence between mammalian and non-mammalian STOPs begins exactly after the last aa of Mn2 (aa 175, according to rat N-STOP sequence) and ends exactly at the first aa located downstream of the central repeats (aa 451, according to rat N-STOP sequence). Between these precise limits, none of the avian, fish, and mammalian STOP protein sequences are related. Thus N-STOP has a dual composition, containing a variable domain inserted between highly conserved domains. In mammals, the inserted variable domain contains Mc modules and has functional properties (calmodulin binding and microtubule stabilization) in common with the conserved domains of N-STOP. This indicates intriguing functional convergence of the variable and conserved domains of STOP proteins in mammals.

**Discussion**

STOP proteins have originally been identified as microtubule-stabilizing proteins whose activity was inhibited by Ca\(^{2+}\)-calmodulin (14–15) and were later shown to be bona fide calmodulin-binding proteins (14–16, 18). The microtubule stabilizing and calmodulin binding activities of STOP now appear to involve multiple microtubule-stabilizing and calmodulin-binding sequences. Motif repetition is a common occurrence in cytoskeletal proteins (32–35) but its significance is not fully understood. One possibility is that repeated motifs allow efficient protein-protein interactions using several clustered low affinity sites instead of using a single high affinity site. This could be important for rapid and flexible regulations, particularly in the case of protein-polymer interactions.

**Mapping of Calmodulin-binding Sites and of Microtubule-stabilizing Domains on N-STOP**—We have used immobilized peptide arrays to characterize calmodulin-binding sites on N-STOP and found 12 distinct calmodulin-binding peptides (Cam1 to Cam7, CamR1 to CamR5). The peptide array technique proved to be useful since most of the 12 peptides did not match known consensus calmodulin binding sequences (36) but proved to be important for STOP function. Thus, the multiplicity of calmodulin-binding sites suggested by immobilized peptide array analysis was confirmed by subsequent Scatchard analysis, which also showed micromolar \(K_d\) values as previously observed for other calmodulin targets (37). Furthermore, eight of the peptides (Cam2, Cam3, Cam5, and CamR1 to CamR5) both interacted with calmodulin and were critically involved in microtubule stabilization.

A striking result of the mapping of the calmodulin-binding and microtubule-stabilizing domains of N-STOP was that it revealed a modular organization of the N-STOP microtubule-stabilizing domains, involving two kinds of repeated motifs, Mn and Mc modules. Although the precise mechanisms through which STOP modules interfere with microtubule stability remain to be determined, these modules are clearly central for STOP activity and their characterization should help to solve fundamental questions concerning the specificity, variations, and regulation of the STOP microtubule stabilizing activity. STOP is a highly basic protein, harboring peptide stretches of pi over 9.0, corresponding, for example, to the exon 3 sequence of the protein (38). Basic proteins can stabilize microtubules through apparently nonspecific ionic interaction and it could have been the case that microtubule stabilization by STOP was due to a general charge effect. In fact, STOP modules occupy limited regions of the protein and STOP fragments devoid of both Mn and Mc modules do not show detectable microtubule stabilizing activity, regardless of their basicity. On the contrary, STOP fragments harboring either type of modules have microtubule stabilizing activity and this activity is determined by their module composition. Fragments containing Mn modules induce microtubule resistance to both the cold and nocodazole, whereas fragments containing Mc modules induce microtubule cold stability only. Interestingly, these different patterns of microtubule stabilization correspond to functional differences previously observed with STOP variants. The neuronal variants N-STOP and E-STOP, which contain all Mn modules, induce resistance to both the cold and nocodazole in cytoplasmic microtubules (12). The fibroblastic variant F-STOP which is principally composed of Mc modules only induces cold stability in cellular microtubules (13). Thus, there is good correlation between the activity of STOP variants and their module composition.

Although global figures are suggestive, the difference between the module composition of STOP variants is not absolute since F-STOP contains Mn3 modules and since neuronal STOPs contain Mc modules in addition to the three Mn modules. The co-existence of both types of STOP modules in a single protein clearly introduces functional interference between modules. Thus F-STOP behaves as Mc modules despite the presence of Mn3 modules. Similarly, when transfected in HeLa cells, STOP subdomains containing only Mn modules (LNt and LCo) induce microtubule resistance to nocodazole in the vast majority of transfected cells (this study) whereas the complete N-STOP, which, additionally contains Mc modules, induces nocodazole resistance in a limited subpopulation of transfected cells (Ref. 18 and this study). Therefore the presence of Mc modules in N-STOP most probably interferes with the regulation of Mn modules.

STOP modules are bifunctional, associating overlapping calmodulin-binding and microtubule-stabilizing sequences. Such bifunctionality suggests that the inhibition of STOP activity by calmodulin is direct, involving a steric inhibition of STOP interaction with microtubules by calmodulin. There are, however, calmodulin-binding peptides in STOP that are not associated with microtubule-stabilizing sequences. This is the case of peptide Cam1 which is apparently involved in STOP interaction with immobilized calmodulin but does not overlap with microtubule-stabilizing sequences in N-STOP. The interaction of calmodulin with peptide Cam1 could still interfere with the microtubule stabilizing activity of N-STOP through steric hindrance or through long range-induced conformational changes. Alternatively, peptide Cam1 may mediate calmodulin-dependent regulation of N-STOP interaction with partners other than microtubules.

Peptides Cam4, Cam6, and Cam7 were detected on immobilized peptide arrays but their contribution to known STOP properties could not be established. These peptides may not be functional in the protein context. However, the functional assays used in the present study are not exhaustive: our assays detect functionality only for peptides localized within microtubule stabilizing domains and/or mediating STOP binding onto immobilized calmodulin in the particular buffer conditions previously used for the complete N-STOP. Therefore further research may reveal a function for these orphan peptides.

**STOP in Evolution and STOP-like Proteins**—A further significant consequence of the characterization of STOP modules was that it suggested clues for analysis of inter-specific structural variations of STOP which looked intriguing. In the present study, a comparison of mammalian STOP sequences with avian and fish sequences has shown that N-STOP comprises a highly conserved domain and a variable domain, inserted at a precisely defined location within this conserved domain. The aa sequences of the variable domain of STOP are highly divergent between mammals, fish, and birds. It is hard to imagine that such a high and precisely localized divergence can arise from...
mutations in a common ancestor gene. Instead, it may correspond to the insertion in an ancestor N-STOP exon 1, of sequences originating from other genes. However, plausible molecular mechanisms for such an insertion remain to be defined. The conserved domain of N-STOP contains peptide stretches comprising Mn modules, capable of inducing microtubule resistance to both the cold and nocodazole. This suggests that the conserved N-STOP domain is sufficient for the stabilization of neuronal microtubules. However, in mammals, this N-STOP domain is associated with a variable domain containing Mc modules and showing common functional properties (calmodulin binding and microtubule cold stabilizing activity). If this functional convergence is important for N-STOP function one would expect to find calmodulin-binding sites and/or microtubule stabilizing activity in fish and avian variable domains.

In mice, the variable domain of N-STOP containing Mc modules is the main component of F-STOP. Similar non-neuronal STOP variants have been detected in rat (12, 13). Putative non-neuronal STOP variants in rodents are also detected in humans (14). The existence of the STOP-like protein SL21 suggests that characteristic STOP domains can be shared by independent proteins indicating that STOP modules and calmodulin-binding sites may represent functional motifs shared by different proteins and be essential for the generation of the various patterns of microtubule stabilization observed in cells.

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