Self-Assembly Incompetence of Synemin Is Related to the Property of Its Head and Rod Domains†

Luiza Khanamiryan,‡,§ Zhenlin Li,‡,§ Denise Paulin,§ and Zhigang Xue*,‡,§

UPMC Univ Paris 6, UMR 7079, Paris, France, and CNRS UMR 7079, Paris, France

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ABSTRACT: The mechanisms regulating the intermediate filament (IF) protein assembly are complex and not yet fully understood. All vertebrate cytoplasmic IF proteins have a central α-helical rod domain flanked by variable head and tail domains. The IF protein synemin cannot homopolymerize to form filament networks; it needs an appropriate copolymerization partner. To elucidate the roles of the vimentin head domain, the TAAL motif in the 2A region, and the TYRKLLEGEE motif in the 2B region of the rod domain in synemin filament formation, we have prepared a series of synemin constructs by site-directed mutagenesis and chimeric synemins having the vimentin head domain. The assembly properties of synemin constructs were assessed by the immunofluorescence of transient transfection into cultured SW13 cells without endogenous IFs. Our data showed that the formation of a filamentous network required at least the vimentin-like head domain and both the 2A and 2B regions of the rod domain.

All vertebrate cytoplasmic intermediate filament (IF)[1] proteins have a central α-helical region of ~310 amino acids flanked by non-α-helical head and tail domains (*1*−*3*). The IF proteins differ mainly in the length of the end domains, which can be as short as eight amino acids at the N-terminus and as long as more than 1200 amino acids at the C-terminus, such as in human nestin (*4*) and synemin (*5*).

The vertebrate IF family can be assigned to six major subfamilies. Some IF proteins, such as vimentin, desmin, peripherin, and glial fibrillary acidic protein (GFAP), can homopolymerize in vivo to form structurally stable and functional IFs (*6*−*9*). Other IF proteins, like keratin types I and II, nestin, and synemin, can form IFs only if they have an appropriate copolymerization partner (*5, 10*−*13*). The mechanisms leading to the polymerization of IF proteins are complex and not yet fully understood. It is certainly not clear why some IF proteins (like synemin) cannot polymerize themselves to form the filament network. Extensive studies of IF assembly in vitro using X-ray crystallography and electron microscopic analysis have shown that IF polypeptides interact via their α-helical rod domains to form filaments 8−10 nm in diameter (*14*−*17*). Despite their differing sequences, the rod domain of all IF proteins contains four α-helix regions with long heptad repeat substructures that are predicted to form coiled coils (*1, 18*); these are designated 1A, 1B, 2A, and 2B. They are separated by short, variable linkers (L1, L12, and L2, respectively). Nearly all IF proteins contain a highly conserved amino acid motif (TYRKLLEGEE) in the 2B region of the rod domain. This motif is crucial for the correct formation of authentic tetrameric complexes and controls the number of subunits per filament cross section during assembly (*15*). A similar sequence containing eight of these ten amino acids (TYRaLLEGEs) is found in the 2B subdomain of the human synemin rod domain (Figure 1). Furthermore, a TAAL motif is perfectly conserved in the 2A subdomain of all group III IF proteins (Figure 1) that can form a homopolymeric filament network. The 2A region of the group IV IF proteins contains a varied motif [(T/S)(A/T/S)AL] [for example, neurofilament L (Figure 1)]. Keratin types I and II have a different motif (Figure 1). However, synemin and nestin do not have this TAAL motif (Figure 1). A few studies have explored the role of the 2A subdomain in assembly of the filament network. The role of the TAAL motif in the 2A subdomain remains unknown. On the other hand, data from numerous in vitro and in vivo experiments suggest also that the head domain of IF proteins is essential for IF formation (*19*−*21*). Consequently, it may be important to elucidate how the absence of the head domain and the different sequences in the 2A and 2B regions of the rod domain influence IF formation. This, in turn, may indicate why synemin does not form filaments.

To examine the roles of the vimentin head domain, the TAAL motif in the 2A region, and the TYRKLLEGEE motif in the 2B region of the rod domain in the filament formation of different synemin isoforms, we have prepared a series of constructs by site-directed mutagenesis and chimeric syn-

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* To whom correspondence should be addressed: CNRS UMR 7079, UPMC Univ Paris 6, 7 Quai Saint Bernard, 75252 Paris, France. Telephone: 33 1 44275438. Fax: 33 1 44272135. E-mail: zhigang.xue@upmc.fr.

‡ UPMC Univ Paris 6, UMR 7079.

§ CNRS UMR 7079.

[1] Abbreviations: IF, intermediate filament; GFAP, glial fibrillary acidic protein; Hu, human; syn, synemin; nes, nestin; K5, keratin 5; K14, keratin 14; vim, vimentin; des, desmin; per, peripherin; NF, neurofilament; int, α-internexin; Syn H (M, L), human synemin H (M, L) isoform; Vh, head domain of synemin replaced with the head domain of human vimentin; Syn 2A, mutation in the 2A region of synemin; Syn 2B, mutation in the 2B region of synemin; Syn 2AB, double mutations in both the 2A and 2B regions of synemin.

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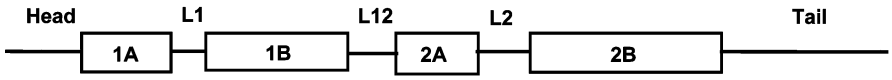
A

	2A	L2	2B
Hu syn	RL....REVHDSYALLVAE	SWRETVQL	YEDEVRELEEALRRGQESRLQAEE
Hu nes	P.....--EE.L-RRLG-	A--GA-RG	-QER-AHM-TS-DQTR-RLAR-VQ
Hu K5	D-**DSII**A--KAQ-EEIANR	-RT-AESW	-QTKYE--QQTAG-HGDDLRNTKH
Hu K14	D-**SRIL**N-MR-Q-EKMAEK	NRKDAEEW	FFTKTE--NREVATNS-LVQSGKS
Hu vim	D-**TAAL**-D-RQQ-ESVA-K	NLQ-AEEW	-KSKFAD-S--AN-NNDALR--KQ
Hu des	L-**TAAL**-DIRAQ-ETIA-K	NIS-AEEW	-KSK-SD-TQ-ANKNNDALR--KQ
Hu per	E-**TAAL**-DIRAQ-ESIA-K	NLQ-AEEW	-KSKYAD-SD-ANRNH-ALR--KQ
Hu GFAP	D-**TAAL**K-IRTQ-EAMASS	NMH-AEEW	-RSKFAD-TD-AARNA-LLR--KH
Hu NFL	D-**SAAL**KDIRAQ-EK-A-K	NMQNAEEW	FKSRFTV-T-SAAKNTDAVRA-KD
Hu int	D-**TSAL**--IRAQ-ES-A-K	NLQSAEEW	-KSKFAN-N-QAARST-AIRASR-

	2B
Hu syn	ETRLCAQEAEALRREALGLEQLRARLEDALLRMREEYGIQAEERQRVIDCLEDE
Hu nes	GA-EVRL-LQQ-QA-RG--LER--A--QR-EGRWQ-RLRAT-KF-LAVEA--Q-
Hu K5	-ISEMNRMIQR--A-IDNVKKQC-N-QN-IADAEQRGELALKDARNKLAE--EA
Hu K14	-ISELRRTMQN-EI-LQSQLSMK-S--NS-EETKGR-CM-LAQI-EM-GSV-EQ
Hu vim	-STEYRRQVQS-TC-VDA-KGTNES--RQMRE-E-NFAVE-ANY-DT-GR-Q--
Hu des	-MMEYRHQIQSYTC-IDA-KGTNDS-MRQMRELEDRFASE-SGY-DN-AR--E-
Hu per	-MNESRRQIQS-TC-VD--RGTNEA-LRQ-RELE-QFALE-GGY-AGAAR--E-
Hu GFAP	-ANDYRRQLQS-TCDLES-RGTNES--RQMREQE-RHVRE-ASY-EALAR--E-
Hu NFL	-VSESRRLLK-KTL-IEACRGMNEA--KQ-QELEDKQNADISAM-DT-NK--N-
Hu int	-IHEYRRQLQARTI-LE--RGANES--RQI-ELE-RHAAEVAGY-DS-GQ--ND

	2B
Hu syn	KATLTLAMADWLRDYQDLLQVKTGLSLEVA**TYRALLEGES**NPQ
Hu nes	-QG-GSQI-QV-EGR-Q-AHL-MS----------**T---A-N**SRL
Hu K5	LQKAKQD--RL--E--E-MNT-LA-DV-I----**K-----E**CRL
Hu K14	L-Q-RCE-EQQNQE-KI--D---R-EQ-I----**R-----D**AHL
Hu vim	IQNMKEE--RH--E-----N--MA-DI-I-**---K-----E**SRI
Hu des	IRH-KDE--RH--E-----N--MA-DV-I-**---K-----E**SRI
Hu per	LRQ-KEE--RH--E-----N--MA-DI-I-**---K-----E**SRI
Hu GFAP	GQS-KDE--RH-QE-----N--LA-DI-I-**---K-----E**NRI
Hu NFL	LR-TKSE--RY-KE-----N--MA-DI-I-**A--K-----E**TRL
Hu int	LRNTKSE--RH--E-----N--MA-DI-I-**A--K-----E**TRF

B



1	12	47	57	158	179	194	202	323	H-1565,M-1253,L-339 (Tail H:1243;M:931;L:17)	**syn**
1	9	44	53	154	173	185	193	314	1621	**nes**
1	169	203	216	316	334	352	361	481	590	**K5**
1	116	150	162	262	279	297	306	426	472	**K14**
1	104	139	147	248	264	283	291	412	466	**vim**
1	109	144	152	253	269	288	296	417	470	**des**
1	98	133	141	242	260	279	287	408	470	**per**
1	70	105	113	214	230	249	257	378	432	**GFAP**
1	91	126	136	237	253	272	280	401	543	**NFL**
1	95	130	140	241	260	279	287	408	499	**int**

FIGURE 1: (A) Amino acid sequence comparison of the 2A and 2B subdomains of the rod domains from certain human IF proteins which can or cannot homopolymerize to form filament networks. Amino acids identical to those of human synemin are indicated by dashes; dots within sequences represent gaps. The TAAL motif in the 2A subdomain and the TYRKLLEGEE motif in the 2B subdomain are indicated in bold type. (B) Diagram showing the domains of different human IF proteins. The amino acid residue numbers of the head domain, the rod domain, and the tail domain are indicated. The lengths of three synemin isoform tails are shown in parentheses. Abbreviations: Hu, human; syn, synemin; nes, nestin; K5, keratin 5; K14, keratin 14; vim, vimentin; des, desmin; per, peripherin; NFL, neurofilament L; int, α-internexin. Amino acid sequences are taken from the following: synemin (*5*), nestin (*4*), keratin 14 (*45*), keratin K5 (*46*), vimentin (*47*), desmin (*48*), peripherin (*49*), GFAP (*50*), neurofilament L (*51*), and α-internexin (*52*).

emins having the vimentin head domain with different wild-type or mutated synemin isoforms. The assembly properties of synemin constructs were assessed via an immunofluorescence study of transient transfection into cultured mammalian cells without endogenous IFs. None of the three isoforms (L, M, and H) became organized into filaments in vimentin-free cells, and the formation of a filamentous network required at least the vimentin-like head domain and both 2A and 2B regions of the rod domain.

MATERIALS AND METHODS

Cloning and Mutagenesis. The three full-length human synemin cDNAs (synemins H, M, and L) (GenBank accession numbers AJ310521, AJ310522, and AJ697971, respectively) were inserted into the pcDNA3 eukaryotic expression vector (Invitrogen). Synemin cDNAs were mutated using the Quick-Change mutagenesis kit according to the manufacturer's protocol (Stratagene). The following primers were used: for the TAAL insertion in the 2A subdomain, 5′-CCGCCGCCACGCCTGACTG-CAGCCCTCCGGGAGGTGCACGACAGC-3′ and 5′-G C T G T C G T G C A C C T C C C G G A G G G C T G-CAGTCAGGCGTGGCGGCGG-3′; for TYRKLLEGEE mutation in the 2B subdomain, 5′-GAGGTGGCGAC-C T A C A G G A A G T T A T T G G A A G G A G A A-GAGAATCCACAGATAGTG-3′ and 5′-CACTATCT-GTGGATTCTCTTCTCCTTCCAATAACTTCCTGTAGG-TCGCCACCTC-3′. The human vimentin head domain was cloned using primers 5′-AAGGTACCGCAGCCATGGCCAC-CAGGTCCGTGTCCT-3′ and 5′-GTGGGCCCCTGGTGT-TCTTGAACTCGGTGTTGAT-3′. The synemin head domain was replaced with the vimentin head domain using KpnI and ApaI sites.

DNA Sequencing and Sequence Analysis. Mutant cDNAs were sequenced by the DNA sequencing facility (Genome Express). The DNA sequence data were analyzed using the BLAST program provided by the NCBI server at the U.S. National Institutes of Health. Helical wheel analysis was performed using Helical Wheel Custom Images and Interactive Java Applet.

In Vitro Transfection Studies. These studies were performed using human adrenocortical carcinoma cells (SW13.C2 vim- cells) (*10*) that lack cytoplasmic IFs. The SW13 cells were cultured as described previously (*5*) except that the cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum. The wild-type or mutated cDNAs encoding full-length human synemins H, M, and L in the pcDNA3 eukaryotic expression vector were used to transfect SW13 cells with Fugene 6 (Roche) or jetPEI reagent (Polyplus-transfection). The resulting proteins were detected by Western blotting and immunocytochemistry. As a control, the vimentin filament network was checked by antibody against vimentin (Progen).

Production of the Antibody against the Synemin Rod Domain. A cDNA fragment corresponding to the rod domain of human synemin (amino acid residues 11−322) was cloned into a pQE32 plasmid (Qiagen) to study the human synemin L isoform. The antibody anti-synRod was produced as described previously (*5*). The resulting rabbit antiserum was characterized by Western blotting analysis. This anti-synRod antibody recognized human synemin H, M, and L isoforms.

Indirect Immunocytochemistry. The immunocytochemistry was carried out as described previously (*22*). The antibody dilutions were as follows: anti-synemin H/M (*5*), 1:500; anti-synRod, 1:100; anti-vimentin (Progen), 1:500. The secondary antibodies used were a swine FITC-conjugated anti-rabbit serum antibody (1:100) and a goat TRITC-conjugated anti-guinea pig antibody (1:100). Samples were examined and photographed using a laser confocal microscope.

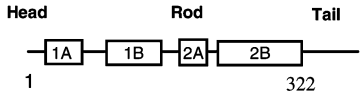
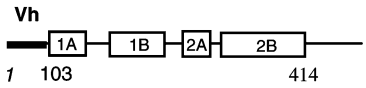
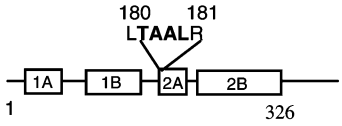
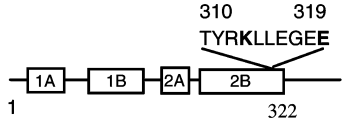
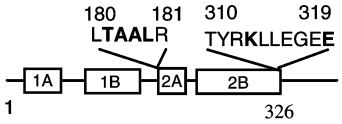
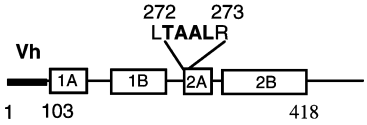
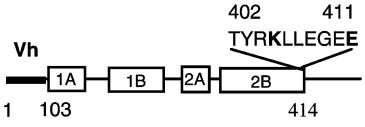
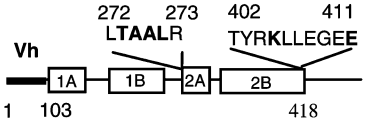
Western Blotting. Total proteins from the transfected cells were prepared as described previously, and immunoblotting was performed (*22, 23*). The primary antibodies were diluted as follows: 1:3000 for rabbit anti-synemin H/M antibody and 1:1000 for rabbit anti-synRod. The reactions were revealed with a peroxidase-conjugated anti-rabbit secondary Ig (DAKO, 1:3000) with an enhanced chemiluminescence detection system (Amersham Biosciences).

RESULTS

On the basis of the evident differences between synemin and type III IF proteins (Figure 1) with regard to the head domain and the 2A and 2B regions of the rod domain, it is important to know whether these differences may influence the IF assembly and network formation. To elucidate the roles of the vimentin head domain, the TAAL motif in the 2A subdomain, and the TYRKLLEGEE motif in the 2B subdomain in filament network formation of the different synemin isoforms, 18 different mutants were designed (see Table 1): group I, mutants of the three isoforms of synemin in which the synemin head domain has been replaced with the vimentin head domain; group II, synemin mutants in the 2A subdomain obtained by inserting a ACTGCAGCCCTC sequence to create the TAAL motif at positions 180 and 181; group III, synemin mutants obtained by changing the TYRaLLEGEs motif to the TYRKLLEGEE motif in the 2B subdomain; group IV, double mutations in both the 2A and 2B domains with an insertion corresponding to the TAAL motif and reconstitution of the TYRKLLEGEE sequence; group V, double mutants with the vimentin head domain and a mutation in the 2A region of the rod domain; group VI, double mutants with the vimentin head and a mutation in the 2B region of the rod domain; group VII, triple mutants with the vimentin head domain and mutations in both 2A and 2B subdomains.

To investigate the synthesis and the difference in the size of wild-type and mutated synemins, synemin cDNAs were transfected into SW13 cells and analyzed by Western blotting with polyclonal antibodies directed against synemin. We detected a 210 kDa band in protein extracts from synemin H, synemin H2A, and synemin H2AB (Figure 2, lanes 1−3). A 170 kDa band was detected in protein extracts from synemin M, synemin M2A, and synemin M2AB (Figure 2, lanes 4−6). Antibodies against the human rod domain detected the 41 kDa protein from transfected synemin L and synemin L2B (Figure 2, lanes 7 and 8). A 51 kDa band was detected in protein extracts from mutated synemin L having a vimentin head domain (Vh-Syn L2A, Vh-Syn L2B, or Vh-Syn L2AB). Figure 2 (lane 9) shows the result for synemin L2B with the vimentin head (Vh-Syn L2B). Antibodies against the synemin detected no band in protein extracts from untransfected SW13 cells (Figure 2, lane 10).

Table 1: Filament Network Formation after Transfection of Different Synemin Constructs in SW13 Cells[a]

	Synemin constructs	Non-filamentous structures	Bundles of filaments	Filament network
	Syn L	+	-	-
	Syn M	+	-	-
	Syn H	+	-	-
I.				
	Vh-Syn L	+	-	-
	Vh-Syn M	+	-	-
	Vh-Syn H	+	-	-
II.				
	Syn L2A	+	-	-
	Syn M2A	+	-	-
	Syn H2A	+	-	-
III.				
	Syn L2B	+	-	-
	Syn M2B	+	-	-
	Syn H2B	+	-	-
IV.				
	Syn L2AB	+	-	-
	Syn M2AB	+	-	-
	Syn H2AB	+	-	-
V.				
	Vh-Syn L2A	-	++	-
	Vh-Syn M2A	-	++	-
VI.				
	Vh-Syn L2B	-	++	-
	Vh-Syn M2B	-	++	-
VII.				
	Vh-Syn L2AB	-	+	++
	Vh-Syn M2AB	-	+	+++

[a] The construction diagrams (wild type and mutants) and the residue numbers of the head domain and the rod domain are shown at the left. The filament formation was visualized by immunofluorescence with anti-synemin H/M antibodies for synemin H and M constructs and the anti-synRod antibody for synemin L constructs. Abbreviations: Syn H (M, L), human synemin H (M, L) isoform; Vh, head domain of synemin replaced with the head domain of human vimentin; 2A, mutation in the 2A region of synemin; 2B, mutation in the 2B region of synemin; 2AB, double mutations in both the 2A and 2B regions of synemin; +, nonfilamentous structures, bundles of filaments, or filament network present; −, nonfilamentous structures, bundles of filaments, or filament network absent.

Synthesis of Synemin Isoforms H, M, and L in SW13 Cells. We have shown that the synemin H and M isoforms alone cannot form filament networks in vitro (*5*). We now find that synemin L like synemin H and M does not form filament networks in vitro (Figure 3A). All three isoforms produced similar patterns, showing the nonfilamentous aggregates (Figure 3A,I,Q and Table 1).

Replacement of the Synemin Head with the Vimentin Head. The head domain plays an important role in the formation of intermediate filaments. The inability of synemin to form

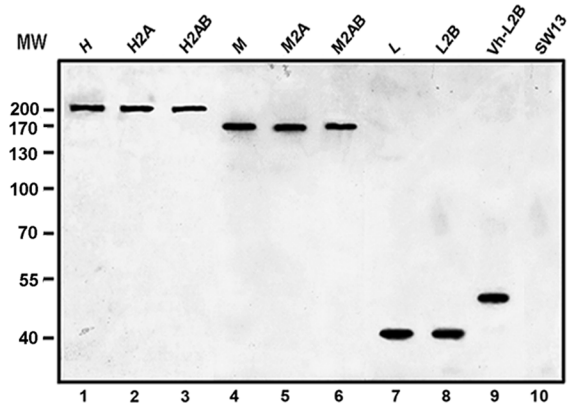


FIGURE 2: Detection of wild-type or mutated synemins by Western blotting after transfection of SW13 cells with cDNAs. The proteins extracted from transfected SW13 cel1s were separated by 12% SDS−PAGE. Molecular mass standards are indicated in kilodaltons. A 210 kDa band was detected in protein extracts from synemin H (lane 1) and synemin mutants H2A (lane 2) and H2AB (lane 3). A 170 kDa band was stained in protein extracts from synemin M (lane 4) and synemin mutants M2A (lane 5) and M2AB (lane 6). The 41 kDa protein was detected in extracts from synemin L (lane 7) and synemin mutant Syn L2B (lane 8). A 51 kDa band was detected in protein extracts from synemin double mutant Vh-Syn L2B (lanes 9). No band was detected in extracts of nontransfected SW13 cells (lane 10) with the anti-synRod antibody.

filaments may be due to its very short head domain. We examined the role of the vimentin head domain in synemin polymerization by replacing the head domain of the three synemin isoforms with the head of vimentin (group I, Vh-Syn L, Vh-Syn M, and Vh-Syn H). None of the three synemin mutants formed filaments; nonfilamentous aggregates were present throughout the cytoplasm. However, the distribution of these structures in the cytoplasm slightly differed from that of wild-type synemins. The nonfilamentous structures were more homogeneous in the cytoplasm when the synemin head domains were replaced with the vimentin head domain (Figure 3E,M, results not shown, and Table 1). Hence, the vimentin head domain alone was not sufficient to influence the formation of filaments by synemins.

Effect of Inserting the TAAL Motif. We investigated the function and impact of the TAAL motif in the 2A region of the rod domain by inserting the TAAL motif into the three synemin isoforms (group II, Syn L2A, Syn M2A, and Syn H2A). The results were similar to those obtained with the wild-type synemins. None of the three mutants formed filaments. The transfected cells contained only nonfilamentous aggregates (Figure 3B,J,R and Table 1). Thus, the TAAL motif alone does not modify synemin polymerization.

Reconstitution of the TYRKLLEGEE Motif. We then examined the influence of the TYRKLLEGEE motif, which is not entirely conserved in all synemins. Its impact on filament formation was determined by transfection in vitro with mutants in the 2B subdomain (group III, Syn L2B, Syn M2B, and Syn H2B). Filaments were not formed in these three mutants. The transfected cells contained nonfilamentous aggregates (Figure 3C,S and Table 1) or diffuse structures (Figure 3K). This result was similar to that obtained with wild-type synemin. Therefore, the TYRKLLEGEE motif alone does not influence the formation of synemin filaments.

Cells Transfected with both TAAL and TYRKLLEGEE Mutations. Since a single mutation did not influence the formation of filaments of synemins, we used combinations of two mutations. We first checked the influence of a combination of TAAL and TYRKLLEGEE motifs on the formation of filament networks (group IV, Syn L2AB, Syn M2AB, and Syn H2AB). We observed a multitude of dots and nonfilamentous aggregates distributed throughout the cytoplasm (Figure 3D,L,T and Table 1). The double mutations in synemin H, M, or L did not influence filament formation.

Contributions of the Head and the 2A and 2B Domains to the Formation of Synemin Networks. Replacing the synemin head domain with the vimentin head did not improve filament formation (Figure 3E,M and Table 1). Single and double mutations with the TAAL motif in the 2A subdomain and the TYRKLLEGEE motif in the 2B subdomain also did not have any influence on synemin polymerization (panels B−D, J−L, and R−T of Figure 3 and Table 1). We therefore produced double or triple mutants of synemins in combination with the vimentin head domain. Double mutants had the head of vimentin and the TAAL sequence in the 2A subdomain (group V, Vh-Syn L2A and Vh-Syn M2A) or the TYRKLLEGEE sequence in the 2B subdomain (group VI, Vh-Syn L2B and Vh-Syn M2B). The triple mutants had the vimentin head, the TAAL sequence in the 2A subdomain, and TYRKLLEGEE mutations in the 2B subdomain (group VII, Vh-Syn L2AB and Vh-Syn M2AB).

SW13 cells transfected with the Vh-Syn L2A and Vh-Syn M2A constructs contained irregularly oriented bundles of filaments (Figure 3F,N and Table 1) but no clear filament networks. Cells transfected with group VI synemin mutant cDNAs containing the head of vimentin and the TYRKLLEGEE mutation in the 2B subdomain formed more regular filaments (Figure 3G,O and Table 1) than did cells transfected with group V constructs (Figure 3F,N). The cells transfected with the synemin M mutant of group VI [Vh-Syn M2B (Figure 3O)] formed longer, more regular filaments than did cells transfected with the synemin L mutant [Vh-Syn L2B (Figure 3G)]. Only those cells transfected with triple mutants having the vimentin head domain, the TAAL motif in the 2A subdomain, and the TYRKLLEGEE mutation in the 2B subdomain contained normal filament networks (Figure 3H,P and Table 1). Approximately 30% of the transfected cells have almost normal networks after being transfected with the triple mutants. No vimentin filament networks were detected in these transfected cells (data not shown). Therefore, the vimentin head domain, the TAAL motif in the 2A subdomain, and the TYRKLLEGEE motif in the 2B subdomain of the rod domain are necessary, but any one alone is not sufficient, for filament network formation. It requires at least these three domains to produce a normal filament network.

DISCUSSION

Most of the many studies on the contribution of IF protein consensus motifs to IF filament network formation published over the past two decades have focused on type III IFs (for reviews, see refs *1* and *18*). These studies have been mainly concerned with the "self-polymerization competent" IFs, like vimentin and desmin. The role of these IF protein consensus motifs was analyzed by removing or modifying the consensus sequences, which resulted in the loss or perturbation of IF

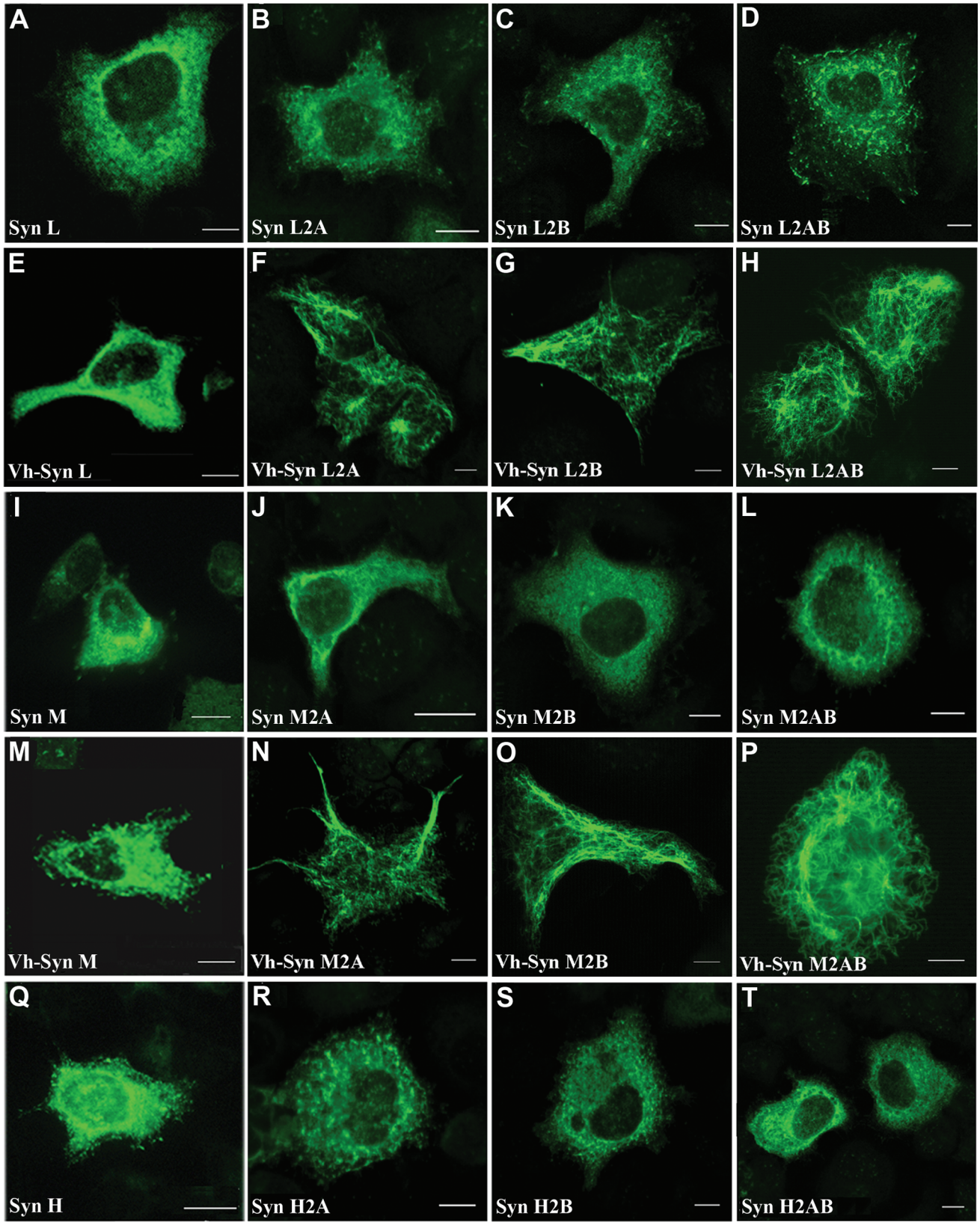


FIGURE 3: Multiple mutations and the formation of synemin networks in vitro. SW13 cells devoid of cytoplasmic IFs were transfected with wild-type or mutated synemin constructs. Wild-type synemin isoforms H, M, and L contained just the nonfilamentous aggregates (A, I, and Q). None of the three isoforms of synemin with mutations in the 2A subdomain (Syn L2A, Syn M2A, and Syn H2A) formed filaments; these cells contained nonfilamentous structures (B, J, and R). Similarly, cells transfected with mutants of synemin in the 2B region of the rod domain (Syn L2B, Syn M2B, and Syn H2B) did not form filaments in vitro (C, K, and S), which was also true for synemin isoforms with mutations in the both domains 2A and 2B (Syn L2AB, Syn M2AB, and Syn H2AB) (D, L, and T). The mutants of synemin isoforms with the head domain of vimentin (Vh-Syn L, Vh-Syn M, and Vh-Syn H) showed only the nonfilamentous aggregates (E and M). Immunofluoresent staining showed that the Vh-Syn L2A (F) and Vh-Syn M2A (N) constructs formed irregular bundles of filaments, but the Vh-Syn L2B (G) and Vh-Syn M2B (O) constructs produced longer, more regular filaments. SW13 cells transfected with the triple mutations Vh-Syn L2AB (H) and Vh-Syn M2AB (P) contained the normal filament networks. The bar is 10 μm.

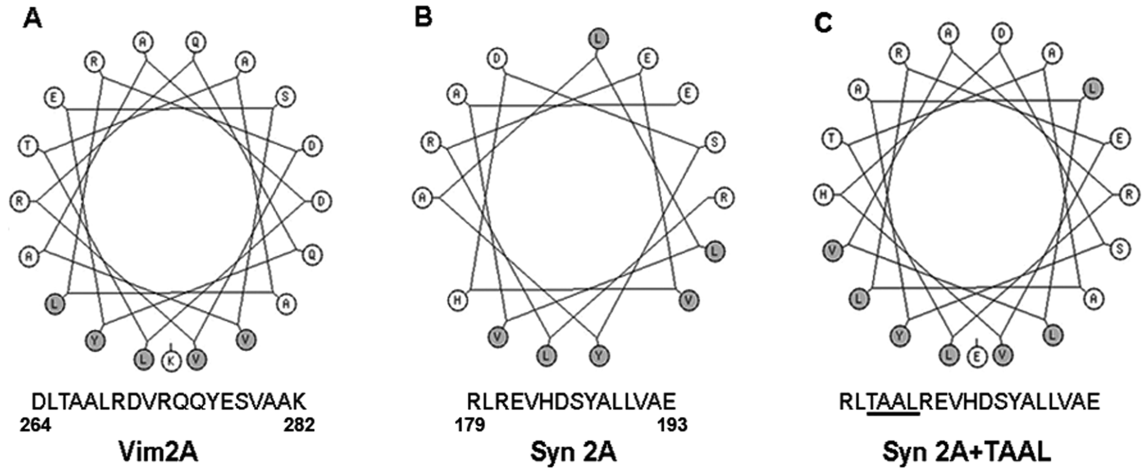


FIGURE 4: Helical wheel projection of the 2A regions of human vimentin and synemin. The corresponding sequences and the residue number are indicated below the picture. Hydrophobic residues are shaded: (A) vimentin wild-type 2A subdomain, (B) synemin wild-type 2A domain, and (C) synemin 2A subdomain with an extra TAAL motif.

network formation. We have now used a "self-polymerization incompetent" IF synemin to identify the contributions of the vimentin head domain, the TAAL motif in the 2A region, and the TYRKLLEGEE motif in the 2B region of the rod domain to IF assembly.

Previous studies have shown that the two end domains modulate or control the assembly of characteristic IFs, at least for the cytoplasmic IF proteins (*24*). The role of the C-terminal tail domain remains more elusive. The head domain is responsible for assembling stable tetrameric complexes, since headless vimentin yields a molecule that did not assemble into 10 nm filaments but remained in a soluble oligomeric particle form (*25*). All IF proteins need the head domain for appropriate dimer−dimer interaction that leads to the formation of unit length filaments (ULFs) (*26*). The rod domain of all the cytoplasmic IF proteins is highly negatively charged at neutral pH, while the N-terminal head domain generally contains few acidic residues but many positively charged arginine residues (12 of 103 in the vimentin head). The head domain of synemin probably does not have enough positively charged residues (1 of 11) to produce this negative and positive balance between the rod and head domains during filament formation, because its head domain is much shorter (11 amino acids) than the head domains of vimentin (103 amino acids), desmin (108 amino acids), or peripherin (97 amino acids). However, the head domain is predominantly hydrophilic, and aromatic amino acids are believed to stabilize three-dimensional protein structures (*27*). The highly conserved nonapeptide motif (SSYRRXFGG) at the very beginning of type III IF protein head domains is critical for IF polymerization (*28*−*30*); changing two of the aromatic residues in the vimentin head to serine makes the IF molecules unable to assemble. Our experiments confirmed that the head domain is necessary for appropriate dimer−dimer interaction that leads to the formation of ULFs (Figure 3). Without the vimentin head domain, the synemins mutated in the 2A and/or 2B region of the rod domain cannot form filaments (panels B−D, J−L, and R−T of Figure 3). On the other hand, our replacement of the head domain of synemins with that of vimentin demonstrated that the head domain alone was not sufficient to influence filament formation. The TAAL motif at the beginning of the 2A subdomain and the TYRKLLEGEE motif at the end of the 2B subdomain of the rod domain are also necessary for normal filament formation and assembly (Figure 3). Clearly, filament assembly needs other elements in addition to the negative and positive balance between the rod and head domains and the role of the nonapeptide (SSYRRXFGG) in the head domain.

The fact that similar results were obtained whatever the length of the synemin C-terminal tail domain suggests that this domain is probably not responsible for the inability of synemin to polymerize. It is also the case in the neurofilament (NF) H and M. It was demonstrated that the long C-termini of NF-H and NF-M are not absolutely required for filament assembly in vivo. The function of the C-terminal tail of NF-M and NF-H appears to be formation of cross bridges to other NFs (*31*−*33*), to mitochondria (*34*), to microtubules (*35*), and perhaps to other cellular structures (*36, 37*). Different studies report that interaction of the long C-terminal tail of synemin with other cytoskeletal components may be a key component linking myofibrillar Z lines to costameres in skeletal muscle cells (*10, 38*−*40*). Synemin H interacts directly by its tail domain with the focal adhesion protein vinculin in its active state. This interaction may anchor the heteropolymer IFs to adhesion-type junctions, such as the costameric regions within striated muscle cells (*40*). The tail domain of synemins H and M was reported to bind to α-actinin, vinculin, dystrophin, and utrophin (*38, 39*). Synemin H and M isoforms can also act as an anchoring protein for protein kinase A (PKA). A putative PKA binding protein (PKAP) domain was identified in the C-terminal tail domain of synemin (*41*).

Strelkov and his colleagues demonstrated that the 1A domain of vimentin forms a single, amphipathic α-helix that probably plays a role in specific dimer−dimer interactions during IF assembly. The 2B domain interferes markedly with IF assembly and also transforms the mature filament into a new kind of structure (*42*). Removing the YRKLLEGEE motif has a profound effect on assembly and changes the lateral packing of dimers (*14, 25*). The X-ray crystallographic study revealed that the atomic structure of the end of the coil 2B domain has 10 amino acids and an "opening" of the coiled coil in the IF consensus motif exposing a cluster of glutamate residues (EGEE) (*15, 42*). This cluster at the end of the coil 2B domain of synemin is modified (EGEs). Our

point mutagenesis experiments showed that this modification can indeed influence tetramer formation and filament polymerization. Synemin double mutants with the vimentin head domain and the TAAL motif in the 2A region of the rod domain, without the TYRKLLEGEE motif in the 2B subdomain, formed only short, irregular filaments (Figure 3F,N). This result confirms the importance of this highly conserved sequence of the rod domain located at the C-terminal end of coil 2B for the proper formation of tetramers and transformation of mature filaments into a network structure. However, the conserved TYRKLLEGEE motif alone is not sufficient for filament polymerization (Figure 3C,K,S). Only cells transfected with synemins having triple mutations (vimentin head, TAAL motif in the 2A domain, and reconstituted TYRKLLEGEE motif in the 2B domain) formed and assembled almost normal filaments (Figure 3H,P).

All IF types have 19 residues in coil 2A. It has an acidic and basic residue period (~9.8 residues long) (*43*). The role of the 2A domain in filament formation and assembly is unknown, but we have shown that the vimentin head domain and a reconstituted TYRKLLEGEE motif in the 2B domain gives filament polymerization (Figure 3G,O). Having TAAL in the 2A domain greatly improves filament assembly (Figure 3H,P). Segment 2A is important for the stability of K5/K14 epidermal keratin filament assembly (*44*). The TAAL motif is highly conserved in the 2A domain of the type III and IV IFs which are able to form homopolymeric filament networks. A possible explanation for the assembly properties of 2A domain is linked to its α-helix structure. Helical wheel analysis with the wild-type vimentin 2A domain shows that the vimentin 2A domain forms an amphipathic α-helix (Figure 4A). This amphipathic structure probably favors the interaction between the 2A domain and other domains to regulate IF protein assembly. The wild-type synemin 2A domain is clearly less hydrophobic, so the synemin 2A domain cannot form an amphipathic structure (Figure 4B). One hypothesis is that inserting an additional TAAL motif into the synemin 2A domain made the mutated synemin 2A domain hydrophobic, resulting in an amphipathic helix structure (Figure 4C) which favors IF assembly. Studies using X-ray crystallography and electron microscopic analysis are now needed to determine the role of the 2A domain.

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