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Phosphorylation-Dependent Control of Structures of Intermediate Filaments: A Novel Approach Using Site- and Phosphorylation State-Specific Antibodies[1]

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Site-specific phosphorylation of intermediate filament (IF) proteins on serine and threonine residues leads to dynamic alterations in filament structure. Site- and phosphorylation state-specific antibodies for IF proteins can visualize spatial and temporal distribution of site-specific IF protein phosphorylations in the cell. These antibodies are also useful to identity IF kinases involved in cellular events, including cell signaling and cell cycle.

Key words: cell cycle, cell signaling, intermediate filament, protein kinases, site- and phosphorylation state-specific antibodies.

Intermediate filaments (IFs) constitute major components of the cytoskeleton and the nuclear envelope in most types of eukaryotic cells (*1, 2*). Although IFs were thought to be relatively stable compared to other cytoskeletons such as microtubles and actin filaments, it has become increasingly evident that site-specific phosphorylation of IF proteins dynamically alters their filament structure. Site-specific phosphorylations are spatially and temporally regulated during cell signaling and cell cycle, and some kinases responsible for the phosphorylation *in vivo* have been identified (see review, *3*). Thus intracellular organization of IF networks is under control of protein kinases and phosphatases. We describe here a brief overview of advances in knowledge concerning the regulation of IF organization and IF kinase activities. We also summarize recent attempts at monitoring site-specific IF protein phosphorylations and at identifying *in vivo* IF kinases during cell signaling and cell cycle. In both cases we utilized site- and phosphorylation state-specific antibodies which are raised against predesigned phosphopeptides and which recognize phosphorylations of IF proteins at specific serine/threonine residues.

In vitro regulation of IFs by site-specific phosphorylation

The first direct evidence that organization of IFs is regulated by phosphorylation was obtained in *in vitro* studies using vimentin (*4*). Vimentin filaments reconstituted *in vitro* underwent complete disassembly when phosphorylated by purified cAMP-dependent protein kinase (A kinase) or protein kinase C (C kinase). Subsequently, similar *in vitro* disassembly induced by phosphorylation was noted for almost all major IF proteins, such as vimentin (*4-14*), glial fibrillary acidic protein (GFAP) (*11, 12*), desmin (*5, 13, 14*), keratin (*15*), α-internexin (*16*), neurofilament (NF)-L (*17-20*), and lamin (*21-26*).

IF proteins are composed of an amino-terminal head, a central rod, and carboxy-terminal tail domains (*2*). Most of the phosphorylation sites of vimentin (*9, 27-30*), GFAP (*11, 12, 31*), desmin (*13, 14, 32*), keratin 8 (*33*), and NF-L (*19, 34*) are located in the head domain and phosphorylation of the head domain is responsible for disassembly of these IFs. On the other hand, NF-H, NF-M, and lamins exhibit characteristics somewhat different from those of above IFs. The tail domains of NF-H and NF-M contain the repeated motif Lys-Ser-Pro or Lys-Ser-Pro-X-Lys that are heavily phosphorylated *in vitro* (*35-38*) and *in vivo* (*35, 39-41*). However, phosphorylation of these sites does not induce disassembly but is considered to regulate the space between individual filaments in the NF fiber network *in vivo*. Phosphorylation sites of lamins by cdc2 kinase are located in both head and tail domains (*21, 42, 43*), and Ser16 of chicken lamin B_2 was shown to be important for lamin head-to-tail polymerization, *in vitro* (*23*).

Site- and phosphorylation state-specific antibodies, a new tool for studying *in vivo* protein phosphorylation events

In 1983, Sternberger and Sternberger reported that a subset of their neuron-specific monoclonal antibodies recognized the specifically phosphorylated form of neurofilaments (NFs) but not the nonphosphorylated forms (Table I). Immunocytochemical staining using these antibodies demonstrated that NFs of certain neuronal cell bodies, dendrites, and proximal axons were nonphosphorylated and those of distal axons were phosphorylated (*44*). Their data were innovative, because they demonstrated that an antibody can distinguish phosphorylated and nonphosphorylated states of a protein and that such a phosphorylation state-specific antibody enables visualization of the intracel-

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TABLE I. **Phosphorylation state-specific antibodies.**

protein	phosphorylation site	name	staining and application	monoclonal/polyclonal	usage	condition	kinase	reference
G substrate	unknown	phospho-antibody	purified protein	polyclonal	IP RI	*in vitro* phosphorylation	G kinase	55
neurofilament (NF)-H	unknown Ser/Thr	04-7	brain homogenate	monoclonal	IB IP IS	terminal exons	unknown	44, 56
NF-H and NF-M	unknown Ser/Thr	05-17	brain homogenate	monoclonal	IB IS	terminal exons	unknown	44
NF-H and NF-M	unknown Ser/Thr	07-5	brain homogenate	monoclonal	IB IS	terminal exons	unknown	44
tau	unknown Ser/Thr				IS	paired helical filaments (PHFs) in Alzheimer's disease		57
NF-H and NF-M	Lys-Ser-Pro motifs in the carboxy-tail domain	SMI-31	brain homogenate	monoclonal	EL IB IS	terminal exons, axonal transection	CDK5 (TPKII)?	58-61
microtubule-associated protein 1B (MAP1B)	unknown Ser/Thr (mode I)				IB IS	neurite outgrowth during brain development	proline-directed protein kinase?	62-64
tau	Ser396 and Ser404				IB IS	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development	MAPK	65-68
NF-H and NF-M	Lys-Ser-Pro motifs in the carboxy-tail domain	SMI-34	brain homogenate	monoclonal	IS	terminal exons	CDK5 (TPKII)?	44, 61
tau	Ser396 and Ser404				IB IS	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development	MAPK	65-68
NF-H	Lys-Ser-Pro motifs in the carboxy-tail domains	SD6	brain extract	monoclonal	EL IB		CDK5 (TPKII)?	58, 61, 69
tau	Ser396				IB IE	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development	GSK-3α, GSK-3β	68, 70-72
NF-H	Lys-Ser-Pro-Val motifs in the carboxy-tail domain (P[+])	Ta51	purified protein	monoclonal	IB IS	axons (+) > perikarya (+) myelination by Schwann cells	CDK5 (TPKII)?	61, 73-75
tau	unknown				IB IS	paired helical filaments (PHFs) in Alzheimer's disease	unknown	74
NF-H	unknown Ser/Thr (P[++])	RMO217	purified protein	monoclonal	IB IS	axons (+) > perikarya (±), myelination by Schwann cells	unknown	73, 75
NF-H	unknown Ser/Thr (P[+++])	RMO24	purified protein	monoclonal	IB IS	axons (+) > perikarya (-), myelination by Schwann cells	unknown	73, 75
NF-M	Lys-Ser-Pro-Val motifs in the carboxy-tail domain (P[+])	RMO93, RMO291	purified protein	monoclonal	IB IS	axons (+) > perikarya (+), myelination by Schwann cells	CDK5 (TPKII)?	61, 73-75
tau	unknown				IB IS	paired helical filaments (PHFs) in Alzheimer's disease	unknown	74
NF-M	unknown Ser/Thr (P[++])	RMO55	purified protein	monoclonal	IB IS	axons (+) > perikarya (±), myelination by Schwann cells	unknown	73, 75
NF-M	unknown Ser/Thr (P[+++])	RMO45	purified protein	monoclonal	IB IS	axons (+) > perikarya (-), myelination by Schwann cells	unknown	73, 75
NF-H and NF-M	Lys-Ser-Pro-Val motifs in the carboxy-tail domain (P[+])	RMO34, RMO52	purified protein	monoclonal	IB IS	axonal transection	CDK5 (TPKII)?	59, 61, 73, 74
NF-H and NF-M	Lys-Ser-Pro motifs in the carboxy-tail domains	RT97	brain extract	monoclonal	EL IB IS	distal axons, axonal transport, myelination by Schwann cells	CDK5 (TPKII)?	58, 60, 61, 76, 77
tau	unknown Ser/Thr in the amino-terminal domain				IB IS IE	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development	unknown	68, 70, 72, 76
NF-M	unknown Ser/Thr in the carboxy-tail domains	BF10	brain extract	monoclonal	EL IB IS	developing neurites	unknown	76, 77
tau	unknown				IS	paired helical filaments (PHFs) in Alzheimer's disease	unknown	76
NF-H and NF-M	Lys-Ser-Pro motifs in the carboxy-tail domains	1215	brain extract	monoclonal	EL IB		CDK5 (TPKII)?	58, 61, 76
tau	unknown				IB IS IE	paired helical filaments (PHFs) in Alzheimer's disease	unknown	70, 76
MAP1B	unknown	mAb 7-1.1	purified protein	monoclonal	IB IS	neurites and cell bodies of mature PC12 cells	unknown	79, 80
MAP1B	unknown Ser/Thr	MAP 1B-3	purified protein	monoclonal	IB	unknown	unknown	81
NF-H and NF-M	unknown Ser/Thr					unknown	unknown	
MAP1B	unknown Ser/Thr	1B6	purified protein	monoclonal	IB IS	axonal elongation	unknown	82
phenylalanine hydroxylase	Ser16	PH7	purified protein	monoclonal	EL IB	unknown	unknown	83
tau	Ser202 (and Ser199)	AT8	purified protein	monoclonal	EL IB IS IE	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development, biopsy-derived adult brain, mitosis	MAPK	67, 68, 84-88
tau	carboxy-terminal domain	anti-ptau 1	purified protein	polyclonal	IB	fetal or juvenile brain	unknown	89
tau	Ser315	anti-ptau 2	purified protein	polyclonal	IB	fetal or juvenile brain	unknown	89
tau	Thr231	AT180	purified protein	monoclonal	IB IE	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development, biopsy-derived adult brain	MAPK + GSK-3β?	67, 90
tau	Thr181	AT270	purified protein	monoclonal	IB IE	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development, biopsy-derived adult brain	MAPK?	67, 90
GAP-43	Ser41	2G12/C7	purified protein	monoclonal	IB IS	distal axon and growth cone during axonogenesis	C kinase	91
β-type platelet-derived growth factor receptor	unknown Tyr	AbP2	nonphosphorylated peptide	polyclonal	IB IP	PDGF stimulation	auto-phosphorylation	92
epidermal growth factor receptor	unknown Tyr				IB IP	EGF stimulation		93
MAP1B	unknown Ser/Thr (mode I)	antibody 150	nonphosphorylated peptide	monoclonal	IB IS	growth cones and distal regions of developing axons	proline-directed protein kinase?	63, 94
MAP1B	unknown Ser/Thr (mode II)	antibody 125	nonphosphorylated peptide	monoclonal	IB IS	neurite outgrowth during brain development	casein kinase II	63, 64

All numberings for tau protein are according to the longest human tau isoform of 441 amino acids (*95*). Abbreviations for usage: EL, enzyme-linked immunosorbent assay (ELISA); IB, immunoblotting; IE, immunoelectron microscopy; IP, immunoprecipitation; IS, immunocytochemical staining; RI, radioimmunoassay.

lular distribution of protein phosphorylation. Thereafter, similar phosphorylation state-specific monoclonal antibodies against a variety of phosphoproteins including NFs, τ (tau) and microtubule-associated protein 1B (MAP1B) have been produced and characterized (Table I). In most cases, the sites of the phosphorylated epitopes were unknown because the antibodies were produced by immunization with tissue homogenates or purified proteins. In some cases, the epitopes have been identified, however the epitopes as such were results of chance (Table I). The *in vitro* data that IF proteins are phosphorylated by distinct kinases at different phosphorylation sites prompted us to monitor site-specific phosphorylation of IF proteins *in vivo*.

To produce an antibody that recognizes phosphorylation of a protein at a specific site, we were the first to utilize a phosphorylated peptide as an antigen (*45, 46*) (Table II). The phosphorylated peptides contained phosphorylated serine/threonine residues which had been identified as

TABLE II. **Site- and phosphorylation state-specific antibodies.**

protein	phosphorylation site	name	immunogen	mono/polyclonal	usage	condition	kinase	reference
glial fibrillary acidic protein (GFAP)	Ser34	pG2	phosphorylated peptide	polyclonal	IB IS	cleavage furrow in cytokinesis	CF kinase	45
GFAP	Ser8	YC10	phosphorylated peptide	monoclonal	EL IB IS	early mitotic phase	cdc2 kinase	46, 48
GFAP	Thr7	pG1-T	phosphorylated peptide	polyclonal	IB IS	cleavage furrow in cytokinesis	CF kinase	48
GFAP	Ser13	pG1-S	phosphorylated peptide	polyclonal	IB IS	cleavage furrow in cytokinesis	CF kinase	48
synapsin I	Ser9	G-257	phosphorylated peptide	polyclonal	IB	forskolin stimulation	A kinase CaM kinase I	47
DARPP-32	Thr34	mAb 23	phosphorylated peptide	monoclonal	EL IB IP IS	agonist-induced increase in cAMP or cGMP levels	A kinase	96
CREB	Ser133	5322 Ab	phosphorylated peptide	polyclonal	IB	nuclear entry of A kinase	A kinase	97
phospholamban	Ser16	PS-16	phosphorylated peptide	polyclonal	EL IB IS	β-adrenergic stimulation	A kinase	98
phospholamban	Thr17	PT-17	phosphorylated peptide	polyclonal	EL IB	β-adrenergic stimulation	CaM kinase	98
calponin	Thr184	anti phosphorylated calponin antibody	phosphorylated peptide	polyclonal	IB	*in vitro* phosphorylation of free calponin	C kinase	99
tyrosine hydroxylase	Ser40	anti-pTH$_{32-47}$	phosphorylated peptide	polyclonal	EL IB IP IS	agonist-induced increase in cAMP level	A kinase	100
RNA polymerase II	YSPTSPS motifs in carboxy-terminal repeat domain (CTD)	anti-PCTD	phosphorylated CTD fusion protein	polyclonal	IB IP IS	transcription on the developmental and heat-shock puffs	CTD kinases	101, 102
p185$^{neu/erbB-2}$	Tyr1248	apt-1	synthetic phosphopeptide	polyclonal	IB	c-erbB-2 activation	auto-phosphorylation	103
p185$^{neu/erbB-2}$	Tyr1248	anti-Pep(*P*)	synthetic phosphopeptide	polyclonal	IB IS	EGF stimulation	auto-phosphorylation	104
p185$^{neu/erbB-2}$	Tyr1248	PN2A	synthetic phosphopeptide	monoclonal	IB IS	EGF stimulation	auto-phosphorylation	105
tau	Ser396 and Ser404	PHF-1	synthetic phosphopeptide	monoclonal	EL IB IS	paired helical filaments (PHFs) in Alzheimer's disease, fetal brain development, biopsy-derived adult brain, mitosis	MAPK	106-109
β1 integrin	Tyr of cytoplasmic domain	anti-PYβ1	synthetic phosphopeptide	polyclonal	EL IP IS	podosomes of RSV-transformed cells	p60^{v-src}?	110
insulin receptor	Ser1327	anti-*P*S1327	synthetic phosphopeptide	polyclonal	EL IB	phorbol ester stimulation	C kinase	111
insulin receptor	Thr1348	anti-*P*T1348	synthetic phosphopeptide	polyclonal	EL IB	phorbol ester stimulation insulin stimulation	C kinase	111
vimentin	Ser55	4A4	synthetic phosphopeptide	monoclonal	EL IB IS IE	early mitotic phase	cdc2 kinase	50
vimentin	Ser82	MO82	synthetic phosphopeptide	monoclonal	EL IB IS IE	Ca^{2+} signaling	CaM kinase II	51
vimentin	Ser33	YT33	synthetic phosphopeptide	monoclonal	EL IB IS	membrane reorganization during mitosis	C kinase	51, 52
vimentin	Ser50	TM50	synthetic phosphopeptide	monoclonal	EL IB IS	membrane reorganization during mitosis	C kinase	52
keratin 18	Ser52	3055	synthetic phosphopeptide	polyclonal	IB IS	S and G2/M phases	unknown	112
AMPA-type glutamate receptor (GluR)	Ser696 of GluR2	12P3	synthetic phosphopeptide	polyclonal	EL IB IS IE	AMPA stimulation in postsynaptic densities for parallel fiber terminals	unknown	113
GFAP	Ser13	KT13	synthetic phosphopeptide	monoclonal	IB IS	cleavage furrow in cytokinesis	CF kinase	54
GFAP	Ser34	KT34	synthetic phosphopeptide	monoclonal	IB IS	cleavage furrow in cytokinesis	CF kinase	54

All numberings for tau protein are according to the longest human tau isoform of 441 amino acids (*95*). Abbreviations for usage: EL, enzyme-linked immunosorbent assay (ELISA); IB, immunoblotting; IE, immunoelectron microscopy; IP, immunoprecipitation; IS, immunocytochemical staining.

phosphorylation sites, in *in vitro* studies. The production of a site- and phosphorylation state-specific antibody by a phosphorylated peptide has the advantage that a phosphorylation site as an epitope can be predesigned (*45-48*) (Table II).

The following is a brief description of our method used to prepare site- and phosphorylation state-specific antibodies. A synthetic peptide that was phosphorylated by protein kinases or a synthetic phosphopeptide served as the antigen. We first used as an antigen the synthetic peptide phosphorylated by several protein kinases (*45, 46*). There is now an established method for synthesizing phosphopeptides, without kinase. Therefore, production of antibodies against such phosphopeptides can now be readily facilitated (Table II). Since 5 or 6 amino acid residues constitute the antigen epitope recognized by the antibody molecule, a peptide consisting of a phosphorylated serine or threonine and its flanking sequences of 5 amino acids (11 amino acids) was designed (*45*). We introduced a cysteine residue at the N or C terminal of the synthetic peptide and bound it to the carrier protein, keyhole limpet hemocyanin (KLH), using maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS). KLH, one of the most commonly used carrier proteins, is effective for antigen presentation necessary for antibody production. We used BDF1 [(C57BL/6×DBA2)F1] mice for immunization, since we find that this F1 hybrid mice produces a larger amount of antibodies against vimentin/GFAP phosphopeptides than do other strains.

We asked why the phosphopeptide of vimentin and GFAP readily raise an antibody specific not only for the

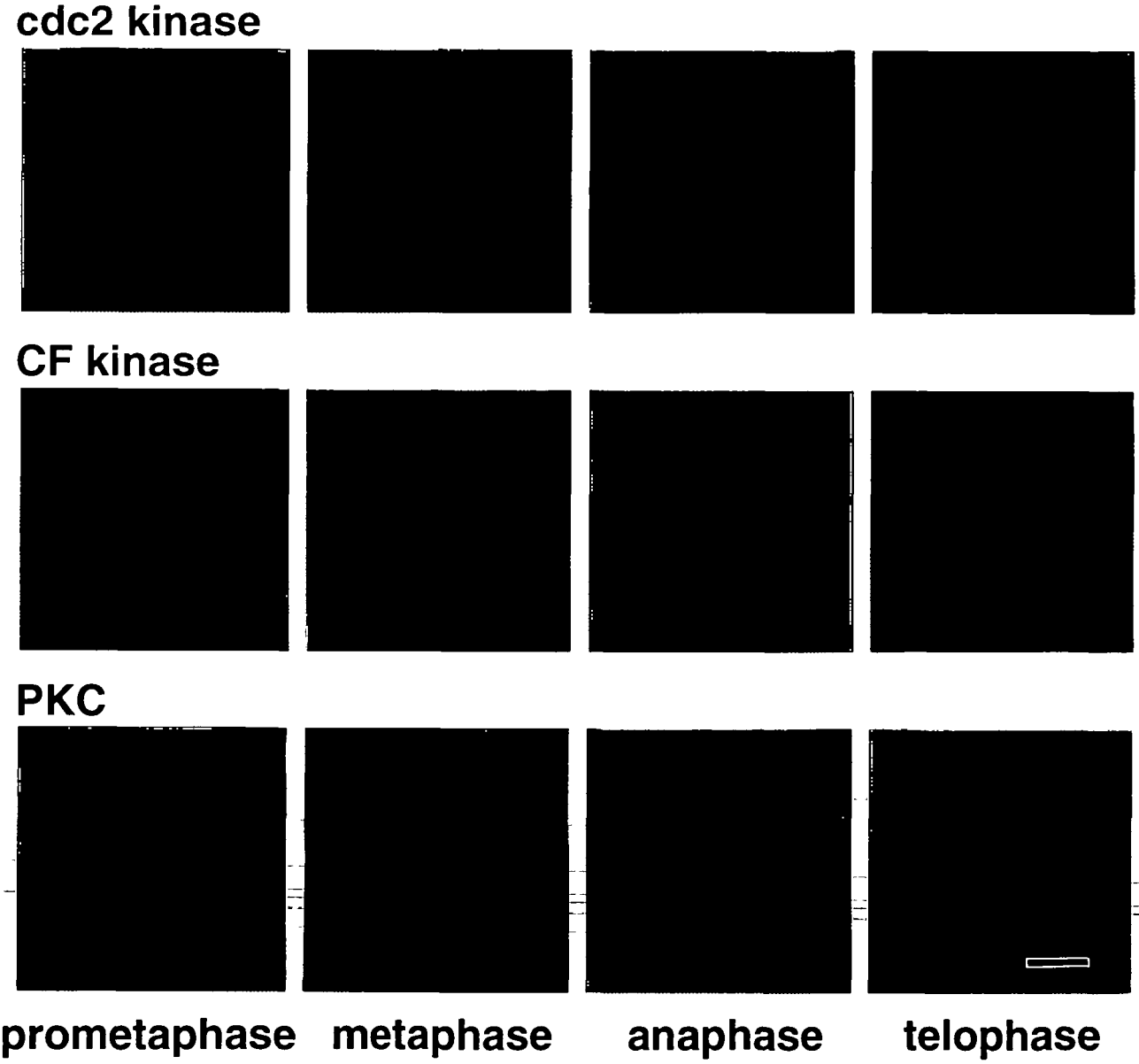

Fig. 1. **Immunofluorescence micrographs of mitotic U251 human glioma cells stained with the antibody 4A4, KT13/KT34, or YT33/TM50 (*50, 52, 54*).** The antibodies 4A4, KT13/KT34, and YT33/TM50 recognize Ser55-phosphorylated vimentin by cdc2 kinase, Ser13-/Ser34-phosphorylated GFAP by CF kinase, and Ser33-/Ser50-phosphorylated vimentin by C kinase, respectively. (Modified with permission, from Refs. *50* and *52*.)

phosphopeptide but also for the native phosphoprotein. This question can be addressed by considering the secondary structure of vimentin and GFAP. The site of phosphorylation is mainly located in the head domain, which is essential for filament formation. Analysis of the secondary structure using the Chou and Fasman method (*49*) revealed that this domain has neither a stable alpha-helix structure nor a beta sheet structure but does have the β turn structure seen in the case of synthetic peptide/phosphopeptide. Such structural homology ensures that an antibody against phosphopeptides of vimentin/GFAP can recognize not only an antigen phosphopeptide but also phosphovimentin/phosphoGFAP.

Detection of *in vivo* IF kinase activities

We utilized the site- and phosphorylation state-specific antibodies to identify *in vivo* IF kinases. Among the *in vitro* phosphorylation sites of IF proteins, there are sites phosphorylated by a single kinase. For example, Ser33, Ser55, and Ser82 residues of vimentin are sites specific for C kinase, cdc2 kinase, and CaM kinase II, respectively (see review, *3*). Such a specific site serves as a pertinent substrate to detect *in vivo* phosphorylation of IF, by a specific kinase. To determine whether cdc2 kinase phosphorylates vimentin *in vivo*, we developed a monoclonal antibody that specifically recognizes phosphorylated vimentin at Ser55 residue (*50*). Ser55 of vimentin was phosphorylated in various types of cells during early mitotic phases (Fig. 1) and chromatographical analysis of mitotic cell lysates revealed a single peak of vimentin-Ser55 kinase activity that is identical to cdc2 kinase (*50*). These data indicate that cdc2 kinase directly and specifically phosphorylates vimentin during early mitotic phases.

Immunocytochemical studies using two monoclonal antibodies that specifically recognize phosphorylated vimentin at Ser33 and Ser82, respectively, revealed differential phosphorylation of vimentin by C kinase and CaM kinase II during cell signaling and cell cycle (*51, 52*). Receptor-mediated phosphoinositide hydrolysis in differentiated astrocytes led to activation of both C kinase and CaM kinase II, but vimentin was phosphorylated only by CaM kinase II, not by C kinase (*51*). CaM kinase II phosphorylates vimentin when activated by Ca^{2+} signaling (*51*). Moreover, our recent studies revealed that Ca^{2+} signaling in a localized area of an astrocyte induced vimentin phosphorylation by CaM kinase II, in the same restricted area, not in other regions of the cell (*53*).

We found the *in vivo* phosphorylation of vimentin by C kinase specifically in mitotic cells but not in interface and differentiated cells (Fig. 1) (*52*). An activator of C kinase, phorbol ester, enhances vimentin phosphorylation by C kinase exclusively in mitotic cells and disrupting the organization of intracellular membranes of interphase cells led to vimentin phosphorylation by C kinase. Therefore, we assume that C kinase phosphorylates vimentin, concomitant with intracellular membrane reorganization during mitosis (*52*). Thus, vimentin phosphorylations by C kinase and CaM kinase II are separately regulated, by distinct mechanisms.

Site- and phosphorylation state-specific antibodies are also useful to detect unidentified *in vivo* IF kinase activities. When an antibody recognizes the phosphorylation of a serine/threonine residue which is commonly the target of redundant kinases, it may detects an unidentified IF kinase activity (*45, 48*). Ser13 and Ser34 residues of GFAP are phosphorylated by redundant kinases *in vitro*. We recently developed monoclonal antibodies which recognize phosphorylated GFAP at these sites. Immunocytochemical studies using these monoclonal antibodies revealed a kinase activity that phosphorylates GFAP in the cleavage furrow (Fig. 1) (*54*). The kinase phosphorylates also ectopically expressed GFAP of non-glial cell at the cleavage furrow and was activated specifically during metaphase-anaphase transition (*54*). Purification and identification of the kinase named cleavage furrow kinase (CF kinase) is ongoing and we will investigate physiological functions and mechanisms of activation at the onset of anaphase.

Conclusions

The present review concerns two major advances in the field of IFs. First, the IF structure was shown to be regulated by phosphorylation and dephosphorylation of their constitute proteins; second, we have established a method to produce site- and phosphorylation state-specific antibodies for phosphoproteins such as phosphovimentin and phosphoGFAP. Phosphorylation and dephosphorylation of proteins dynamically alter their structures and related functions. Therefore, detection and visualization of the site-specific protein phosphorylation will reveal unknown mechanisms governing the regulation of wide-ranged cellular activities. The site- and phosphorylation state-specific antibody we have described here is expected to have a wide application.

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