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Vimentin Serves as a Phosphate Sink During the Apparent Activation of Protein Kinases by Okadaic Acid in Mammalian Cells

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Abstract The vimentin contents of four mammalian cell lines originating from rat and human tissues were determined by immunoblotting and scanning densitometry. On per cell volume basis, vimentin content in 9L, KD, and HeLa cells was found to be 206.6, 151.6, and 19.1 ng/μl, respectively. A431 cells were devoid of vimentin. Protein phosphorylation was augmented by treatment of 600 nM okadaic acid for 1 h in these cells. During the apparent activation of protein kinases, vimentin became hyperphosphorylated and the phosphorylation level of other nonvimentin phosphoproteins was relatively little affected in 9L and KD cells. In contrast, cytokeratins and other nonvimentin proteins were heavily phosphorylated in OA-treated HeLa and A431 cells. Regression analysis indicated that the relative increase in phosphorylation level of nonvimentin phosphoproteins was inversely correlated to the contents of vimentin in the four cell lines [$r^2 = -0.985$]. These observations strongly suggest that vimentin acts as a phosphate sink by which the effects of "excess kinase activity" inflicted by phosphatases inhibition was attenuated. © 1993 Wiley-Liss, Inc.

Key words: vimentin, intermediate filament, protein phosphorylation, immunoblotting, scanning densitometry

Cytoplasmic IF network is a major cytoskeletal system with a diameter of 8 to 12 nm found in a wide variety of mammalian cells [Traub, 1985; Steinert and Roop, 1988; Goldman and Steinert, 1990]. It is well established that the constituent polypeptides of IF are part of a large family sharing common structural domains [Bloemendal and Pieper, 1989; Steinert and Roop, 1988; Goldman and Steinert, 1990]. Based on sequence homology and molecular structure, 6 categories have thus far been identified [Lendhal et al., 1990; Skalli and Goldman, 1991]. The expression of IF proteins is tissue and cell type specific [Traub, 1985; Klymkowsky et al., 1989]. In cells of mesenchymal origin and most cells in culture, IF is mainly composed of vimentin [Georgatos and Blobel, 1987]. In contrast, cytokeratins are the major IF proteins in cells of epithelial origin [Moll et al., 1982; Schliwa, 1986]. Specific cells in culture [e.g., HeLa] contain both vimentin and cytokeratin IF systems [Franke et al., 1979a,b]. IFs form a network interconnecting the nuclear envelope and the plasma membrane [Georgatos and Blobel, 1987]. Together with IF associated proteins [Steinert and Roop, 1988; Yang et al., 1990], the cytoplasmic IF and the nuclear IF may act in concert to convey mechanical as well as molecular information from the cell surface to the nuclear and vice versa [Goldman et al., 1985, 1986; Skalli and Goldman, 1991]. However, the physiological function[s] of IF is not yet fully understood [see Geiger, 1987; Bloemendal and Pieper, 1989; Skalli and Goldman, 1991].

Post-translational modifications of vimentin are common phenomena which may be involved in the structural organization of the vimentin IF. A small portion of vimentin is found to be phosphorylated in quiescent cells [Cabral and Gottesman, 1979; Nelson and Traub, 1983; Lee et al., 1992] and all of the phosphovimentin molecules, which are not extractable by nonionic detergents, are located in the cytoskeleton-nuclear fraction [Lee et al., 1992]. Two-dimensional gel analysis of proteins from [^{32}P]orthophosphate-labeled cells has revealed that phosphorylated vimentin is among the most prominent phosphoproteins in the cytoplasm

Abbreviations used: IF, intermediate filament; CK, cytokeratin; OA, okadaic acid; SDS-, 2D-PAGE, sodium dodecylsulfate-, two-dimensional polyacrylamide gel electrophoresis.

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[O'Connor et al., 1981; Spruill et al., 1983; Tsuda et al., 1988]. The phosphorylation of vimentin has been demonstrated to be site-specific and in most cases restricted to the 9 kDa N-terminal domain [Inagaki et al., 1987; Geisler et al., 1989; Ando et al., 1989]. The process appears to be involved in the reorganization of the vimentin filaments [Inagaki et al., 1987; Lamb et al., 1989; Chou et al., 1989; Geisler et al., 1989] which has been shown to be altered dramatically during mitosis [Celis et al., 1983; Franke et al., 1984; Chou et al., 1989], as well as during treatments with hormones [Spruill et al., 1983; Coca-Prados, 1985], heat [Thomas et al., 1982; Welch and Suhan, 1985; Welch et al., 1985], and OA [Lee et al., 1992]. During the above treatments, vimentin IF usually switches from filamentous form to a higher order of organization [see Lee et al., 1992].

Okadaic acid (OA), a toxin isolated from a variety of marine species [Tachibana et al., 1981], is a potent inhibitor of a number of protein phosphatases including type 1 and type 2A protein phosphatases [Bialojan and Takai, 1988]. The inhibitory effect blocks the dephosphorylation of proteins that are substrates for multiple protein kinases [Suganuma et al., 1989], resulting in the apparent "activation" of the kinases [Issinger et al., 1988; Sassa et al., 1989] and subsequent induction of a variety of cellular responses that are modulated by protein phosphorylation-dephosphorylation [Haystead et al., 1989; Yamashita et al., 1990]. In a recent study, we found that the phosphorylation level of vimentin is drastically elevated by OA in a reversible manner and that the hyperphosphorylation of vimentin could result in the reorganization of the vimentin IF which is more detergent-extractable [Lee et al., 1992]. We have further characterized the involvement of IF components during OA induction of hyperphosphorylation of intracellular proteins using four different cell lines containing different amounts of vimentin or kinds of IF components. Our results indicate that vimentin, and possibly other IF proteins, are preferentially hyperphosphorylated during the apparent activation of protein kinases in the presence of OA. These results suggest that the IF components may act as a phosphate sink during an inflicted disturbance of the protein kinases-phosphatases balance in cells.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise indicated, chemical reagents were obtained from either Sigma or Merck. [^{32}P]orthophosphate (specific activity 8,500–9,120 Ci/mmol) was obtained from Du Pont-New England Nuclear. Culture medium components were purchased from Gibco Laboratories and supplies for immunostaining were purchased from Amersham.

Cells and Cell Culture

Rat gliosarcoma 9L cells were a gift of Dr. M.L. Rosenblum (University of California at San Francisco) and were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin [Weizsaecker et al., 1981]. KD (human fibroblast), HeLa (human cervical carcinoma), and A431 (human epidermoid carcinoma) cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) containing the same supplements. Cell numbers were determined by a haemocytometer. Exponentially growing cells at 70–90% confluency were used.

Electrophoresis, Western Blotting, and Autoradiography

SDS-PAGE and 2-D PAGE were performed according to the methods of Laemmli [1970] and O'Farrell [1975], respectively. Sample preparation and other related experimental procedures were carried out as previously described [Lee et al., 1991, 1992; Hou et al., 1993].

Quantitative Analysis of Cellular Vimentin

Cell lysates from known volume and number of cells were electrophoresed along with serially diluted pure vimentin. After electrophoresis and Western blotting, the optical densities of the vimentin bands were determined and the vimentin contents of the cells were calculated from the calibration curve constructed from the pure samples.

Labeling With [^{32}P]orthophosphate and Treatment With Okadaic Acid

For in vivo labeling of proteins with [^{32}P]orthophosphate, cells were washed twice with phosphate-free medium and labeled for 1 h. Cells

Table I. Amounts of Vimentin in 9L, KD, HeLa and A431 Cells*

Cell lines	ng/μl	Molecules/cell
9L	206.6 ± 9.2	$6.7 \pm 0.3 \times 10^8$
KD	151.6 ± 5.6	$2.7 \pm 0.1 \times 10^9$
HeLa	19.1 ± 1.2	$6.5 \pm 0.4 \times 10^7$
A431	Not detectable	Not detectable

*Cells at 70 to 90% confluency were lysed in SDS sample buffer and the lysates were resolved by SDS-PAGE together with serially diluted pure vimentin. After electrophoresis, protein bands were analyzed by Western blotting using mouse antivimentin mAb as the primary antibody and alkaline phosphatase-conjugated goat antimouse IgG antibody as the secondary antibody. Following color development, the vimentin bands on the immunoblots were quantitated by scanning densitometry. Vimentin contents in cells were calculated from the calibration curves constructed from the pure samples. Values are the means ± S.D. obtained from four independent experiments.

were then treated with 600 nM OA for an additional h in the presence of [^{32}P]orthophosphate (1 mCi/ml). After treatment, the cells were washed three times with ice-cold PBS and lysed with sample buffer [Laemmli, 1970] or lysis buffer [O'Farrell, 1975], depending on the electrophoretic system employed. Bands on the X-ray films (Fuji) and Western blots were quantitated by densitometric scanning (Molecular Dynamics).

RESULTS

The vimentin contents of 9L, KD, HeLa, and A431 cells were determined by SDS-PAGE, immunoblotting, and scanning densitometry. On per cell volume basis, 9L, KD, and HeLa cells respectively contained 206.6, 151.6, and 19.1 ng/μl of vimentin. In contrast, vimentin was not

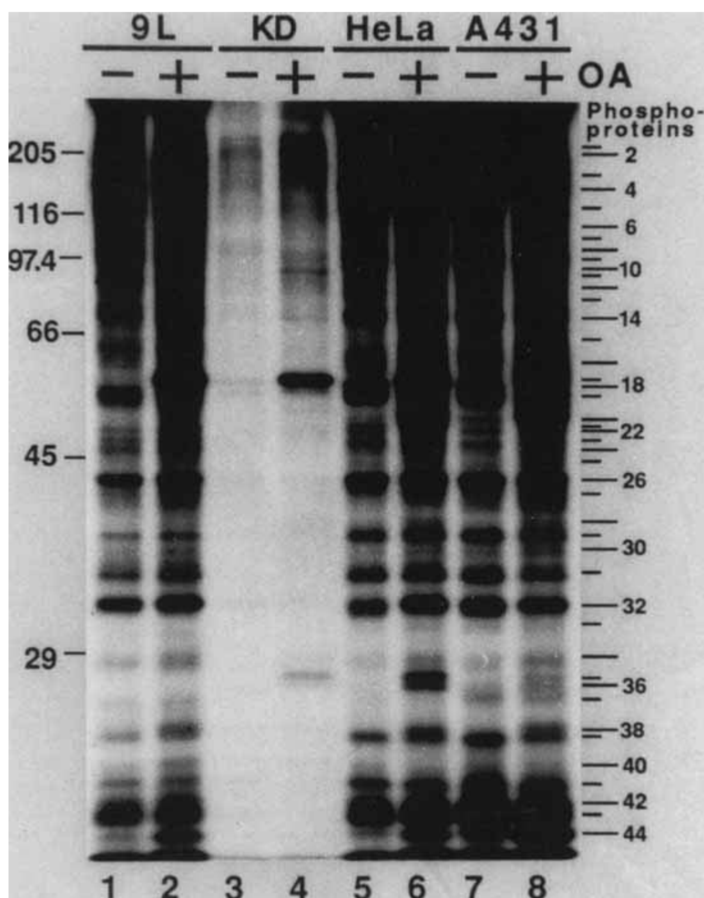

Fig. 1. Effect of OA on the phosphorylation levels of phosphoproteins in 9L, KD, HeLa, and A431 cells. Cells were pre-labeled with [^{32}P]orthophosphate for 1 h and then treated with 600 nM OA for 1 h in the presence of the isotope. The cells were then lysed with SDS sample buffer [Laemmli, 1970] and the lysates were resolved by SDS-PAGE. After electrophoresis, the gels were processed for autoradiography. **Lanes 1, 3, 5, 7:** Untreated cells. **Lanes 2, 4, 6, 8:** OA-treated cells.

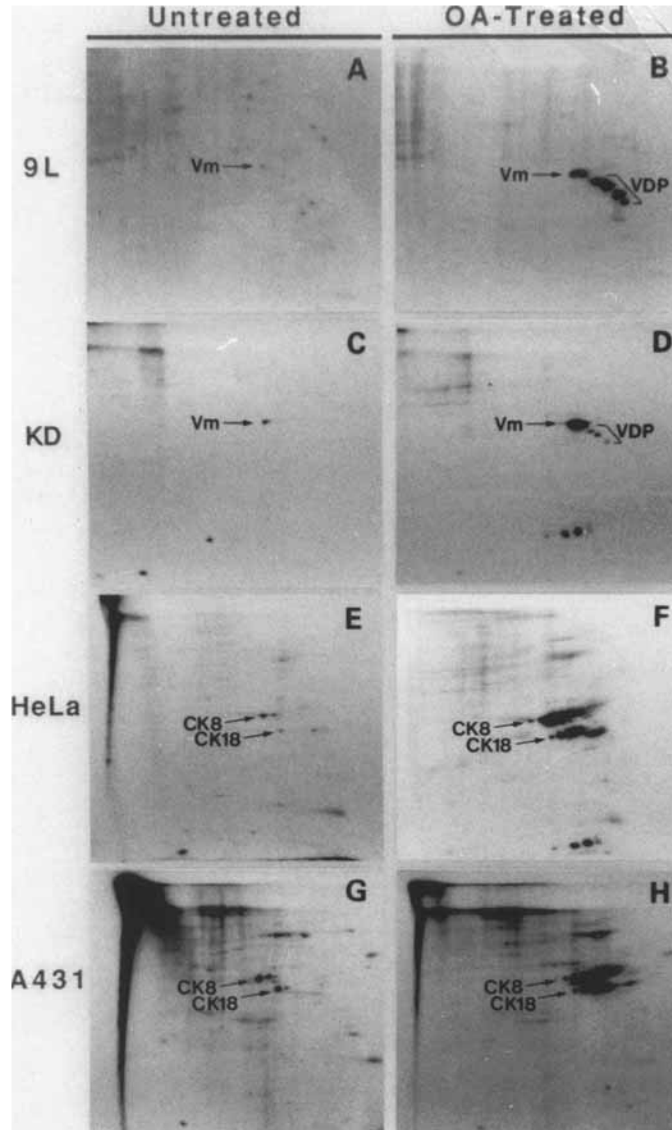

Fig. 2. Two-dimensional electrophoresis of phosphoproteins in OA-treated cells. Cells were treated as described in Figure 1 but the cells were lysed in lysis buffer [O'Farrell, 1975] and the cell lysates were resolved by 2-D PAGE. The IF proteins were identified. Vm, authentic vimentin; VDP, degradation products of vimentin; CK8, cytokeratin 8; CK18, cytokeratin 18.

detectable in A431 cells (Table I). Other IF proteins, such as desmin, glial fibrillary acidic protein, and neurofilament proteins were not detected in these cells. However, a significant amount of cytokeratins (CK8 and CK18) were observed in HeLa and A431 cells but not in 9L and KD cells. Therefore, the cell lines could be roughly divided as vimentin-rich, i.e., 9L and KD cells, vimentin-poor, i.e., HeLa cells, and vimentin-absent, i.e., A431 cells. A431 cells, which are of epithelial origin, contained only cytokeratin IFs. Also note that HeLa cells contained both vimentin and cytokeratins, an observation consistent with that as reported by Franke et al. [1979a,b].

Since OA was identified as potent protein phosphatase inhibitor [Bialojan and Takai, 1988], is has been widely used as a tool to study a variety of cellular processes regulated by protein phosphorylation-dephosphorylation [Sassa et al., 1989; Goris et al., 1989; Picard et al., 1989; Haystead et al., 1989; Cohen et al., 1990; Yamashita et al., 1990; Yatsunami et al., 1991; Lee et al., 1992; Hou et al., 1993]. The above

Table II. Effect of Okadaic Acid on the Phosphorylation Levels of Phosphoproteins in 9L, KD, HeLa, and A431 Cells*

Phosphoproteins		9L	KD	HeLa	A431
1		NC	NC	NC	NC
2		NC	NC	NC	NC
3		ND	NC	NC	NC
4		NC	NC	NC	NC
5		NC	NC	-	NC
6		NC	ND	NC	NC
7		ND	ND	NC	NC
8		NC	ND	NC	NC
9		ND	NC	NC	NC
10		ND	ND	-	NC
11		ND	NC	ND	ND
12		NC	ND	NC	NC
13		+	-	NC	++
14		NC	ND	NC	NC
15		NC	NC	+++	NC
16		ND	ND	NC	+
17	(Vm)	+++	+++	+++	ND
18	(CK8)	ND	ND	+	+
19		NC	++	+	NC
20	(CK18)	ND	++	+++	+++
21		+	ND	++	ND
22		+	NC	+	+
23		NC	ND	NC	+
24		ND	ND	+	ND
25		ND	ND	NC	+
26		NC	NC	NC	NC
27		NC	ND	+	NC
28		NC	ND	NC	NC
29		+	ND	+++	NC
30		+	ND	+	NC
31		NC	-	NC	NC
32		NC	-	+	NC
33		ND	+++	+	+
34		NC	++	++	++
35		ND	+++	+++	+++
36		ND	++	+++	NC
37		NC	ND	ND	NC
38		+	-	+	NC
39		NC	ND	NC	-
40		NC	-	NC	NC
41		NC	-	NC	NC
42		NC	-	NC	NC
43		NC	-	NC	NC
44		+	NC	NC	NC

*Cells were prelabeled with [^{32}P]orthophosphate for 1 h and treated with 600 nM OA for an additional h in the presence of the isotope. Cells were then lysed and the lysates were resolved by SDS-PAGE. Autoradiograms as shown in Figure 1 were quantified by scanning densitometry. The changes in band intensity were matched against their respective controls in the untreated cells and scored: ND, not detected; NC, not changed; −, greater than 50% decrease in protein phosphorylation; +, less than 2-fold increase; + +, 2–5-fold increase; + + +, $>$5-fold increase in protein phosphorylation.

cells were metabolically labeled with [^{32}P]orthophosphate and then treated with 600 nM OA for 1 h. The cells were then lysed and the intracellular proteins were resolved by SDS-PAGE. Phosphoproteins were visualized by autoradiography. Figure 1 shows that the levels of protein phosphorylation were augmented in all treated cells. However, the increase in phosphorylation level appears to be cell and protein specific, dephosphorylation on limited species of protein was also observed. In 9L, KD, and HeLa cells, vimentin was hyperphosphorylated (Fig. 1). Two-dimensional PAGE indicated that the pIs of the authentic vimentin (Vm) and its degradation products (VDPs) were affected, due to increased levels of phosphorylation (Fig. 2A–D). Similar effect was observed for cytokeratins (CK8 and CK18) in HeLa and A431 cells. In addition, a series of isoforms of CKs were generated in these two cell lines (Fig. 2E–H).

Autoradiographs as shown in Figure 1 were quantitated by scanning densitometry and the data were summarized in Table II. In 9L and KD cells, which contain high level of vimentin, the patterns of phosphoproteins were little changed after OA treatment except vimentin, which became hyperphosphorylated (Fig. 1, lanes 1–4; Table II). In contrast, in HeLa and A431 cells, which contain a low level or are devoid of vimentin, the patterns of non-vimentin phosphoproteins (i.e., phosphosproteins except vimentin) were significantly affected. Both CK8 and CK18 in these cells were hyperphosphorylated. In addition, the phosphorylation levels of a number of phosphoproteins in these two cells were also significantly augmented (Fig. 1, lanes 5–8; Table II). The quantitative data on the changes of phosphorylation levels of vimentin and nonvimentin proteins in these four cell lines were presented in Table III. The data indicated that in vimentin containing cells, i.e., 9L, KD, and HeLa cells, the phosphorylation level of this protein increased at least 10-fold after treatment with OA. Furthermore, in OA treated cells, the increase in phosphorylation level of nonvimentin proteins increased as the vimentin contents decreased. Regression analysis demonstrated that the increase in phosphorylation level of nonvimentin phosphoproteins was inversely correlated to the vimentin contents in these cells (Fig. 3).

Table III. Phosphorylation Levels of Vimentin and Nonvimentin Phosphoproteins Affected by Okadaic Acid in 9L, KD, HeLa, and A431 Cells*

Cell lines	Total phosphoproteins		Vimentin		Nonvimentin phosphoproteins		Relative increased phosphorylation of nonvimentin phosphoproteins, %
	−OA	+OA	−OA	+OA	−OA	+OA	
9L	25,493	32,569	163	3,886	25,330	27,683	47.4
KD	8,039	13,923	238	2,743	7,801	11,180	57.4
HeLa	32,470	48,226	174	1,965	32,296	46,301	88.7
A431	35,367	43,762	0	0	35,367	43,762	100.0

*Cells were prelabeled with [^{32}P]orthophosphate for 1 h and treated with 600 nM OA for an additional h in the presence of the isotope. Cells were then lysed and the lysates were resolved by SDS-PAGE. Autoradiograms as shown in Figure 1 were quantified by scanning densitometry and band intensities in total pixels were scored. The summation of band intensities of all identifiable bands in each lane is defined as "total" and the "nonvimentin phosphoprotein" is calculated from the difference between total and vimentin phosphorylation levels. Relative increase phosphorylation of nonvimentin phosphoprotein is expressed as a percentage of the increase in phosphorylation level in nonvimentin phosphoproteins relative to the increase in phosphorylation level in total phosphoproteins.

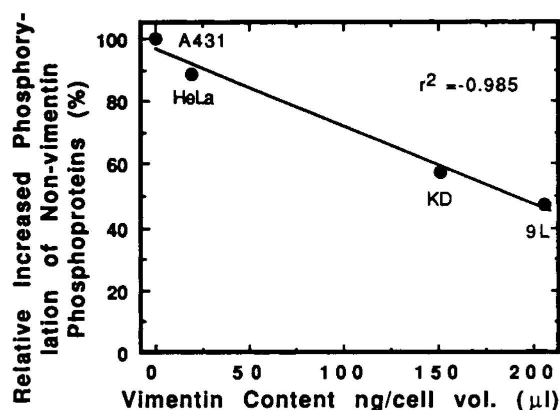

Fig. 3. Correlation of the relative increased phosphorylation of the non-vimentin proteins to the vimentin contents in 9L, KD, HeLa, and A431 cells. Linear regression analysis was performed on the relative increased phosphorylation of the non-vimentin proteins (Table III, last column) and vimentin contents in 9L, KD, HeLa, and A431 cells (Table I).

DISCUSSION

Phosphorylation-dephosphorylation of proteins is one of the major regulatory processes in eucaryotic cells. In a signal transducing pathway, the activation of protein kinases by intracellular second messengers such as cAMP, cGMP, Ca^{2+}, and diacylglycerol is the critical event in producing physiological response. On the other hand, protein phosphatases are involved in the reverse processes [Cohen, 1989, 1992]. It is now well established that both phosphorylation and dephosphorylation are dynamic processes by which the target proteins are flickered on/off by cycling between its phosphorylated and dephosphorylated forms [see Brautigan et al., 1989; Cohen, 1992]. In a resting cell, target proteins are cycled between their phosphorylated and dephosphorylated forms at a certain rate and the process must be maintained in delicate equilibrium. Severe upset of this equilibrium must be avoided and multiple cellular mechanisms must be in place to compensate for imbalances between activities of kinases and phosphatases should this happen.

We have demonstrated that vimentin is preferentially phosphorylated in OA-treated cells containing high levels of vimentin. In contrast, in cells containing low level or devoid of vimentin, other IF proteins becomes the primary target for phosphorylation under OA treatment. Vimentin is a substrate for cdc2-related protein kinase [Chou et al., 1989, 1990, 1991], cAMP-dependent protein kinase [Ando et al., 1989; Lamb et al., 1989], diacylglycerol regulated protein kinase [Huang et al., 1988; Ando et al., 1989], cGMP-dependent protein kinase [Wyatt et al., 1991], as well as Ca^{2+}-calmodulin-dependent protein kinase II [Ando et al., 1991]. All together, a total of more than 15 serine residues are identified as potential phosphorylation sites on this protein [Ando et al., 1989, 1991; Chou et al., 1989, 1990, 1991]. However, under normal conditions, only a small portion of vimentin is phosphorylated despite the presence of many potential phosphoryation sites [Cabral and Gottesman, 1979; Nelson and Traub, 1983; Lee et al., 1992]. Our results showing rapid increases in vimentin phosphorylation in vimentin containing cells in the presence of OA indicate that

the low level of vimentin phosphorylation in the untreated cells is a consequence of high protein phosphatase activity, rather than low kinase activity. Combined with the observation that rapid dephosphorylation occurs upon the removal of the inhibitor [Lee et al., 1992], a high turnover rate of phosphate on vimentin might be postulated. It has been suggested that this high turnover rate may be related to the regulation of IF assembly state and that protein phosphatase activity is required for cytoskeletal integrity in vivo [Eriksson et al., 1992].

Taken together, because of its reactivity to multiple protein kinases, multiple phosphorylation sites, and the virtually reversibility of the process, vimentin [and perhaps other IF components] is a perfect candidate to act as a phosphate sink during a disturbance of protein phosphorylation-dephosphorylation balance in cells.

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