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Phosphorylation of vimentin head domain inhibits interaction with the carboxyl-terminal end of α-helical rod domain studied by surface plasmon resonance measurements

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Abstract The amino-terminal head domain of vimentin is the target site for several protein kinases and phosphorylation induces disassembly of the vimentin intermediate filaments in vivo and in vitro. To better understand molecular mechanisms involved in phosphorylation-dependent disassembly, we examined domain interactions involving the head domain and the effect of phosphorylation on the interaction, using surface plasmon resonance. We observed that the head domain binds to the carboxyl-terminal helix 2B in the rod domain, under physiological ionic strength. This interaction was interfered with by A-kinase phosphorylation of the head domain. Deletion of the carboxyl-terminal 20 amino acids of helix 2B resulted in loss of the interaction. Furthermore, peptide representing the carboxyl-terminal 20 residues of helix 2B had a substantial affinity with the head domain but not with the phosphorylated one. These findings support the idea that the interaction between the head domain and the last 20 residues of helix 2B is essential for association of vimentin tetramers into the intermediate filaments and that the phosphorylation-dependent disassembly is the result of loss of the interaction.

Key words: Intermediate filament; Vimentin; Phosphorylation; Domain interaction; Surface plasmon resonance

1. Introduction

Intermediate filaments (IFs), important constituents of the cytoskeleton of most eukaryotic cells, perform a number of key structural roles in eukaryotic cells and are also thought to be an important determinant of cytoplasmic organization [1–6]. IF subunit proteins are composed with three domain structures [7–10]: an α-helical rod domain of relatively conserved sequences and sizes (about 310 residues), and flanking amino-terminal head and carboxyl-terminal tail domains of variable length, sequences and chemical characteristics. The rod domain can be further subdivided into four helices 1A, 1B, 2A and 2B with short spacers between them (Fig. 1).

Mechanisms leading to IF formation from the subunit proteins are not fully understood [7–10]. The subunit proteins exist as dimers and tetramers at low ionic strength, and spontaneously assemble into IF at higher (physiological) ionic strength [11]. The rod domain is responsible and is sufficient for the initial association of IF subunit proteins into a coiled-coil dimer and then for the association of a two dimer into a tetramer [12–14]. While available evidence indicates that the tail domain is largely dispensable for IF formation [15–17], the head domain (HD) seems to be crucial for IF assembly. IF proteins lacking the HD remain in tetramers and are assembly incompetent [15,18–20]. Moreover, the HD-specific phosphorylation in vitro (for vimentin, see [3,21–26] or in vivo [3,26–31]) leads to disassembly of the filaments. Dephosphorylation of the HD by a phosphatase leads to recovery of the polymerization competence of IF proteins in vitro and in vivo [3,26–30]. However, the exact role of the HD in IF assembly has remained obscure.

Here we show that the HD of vimentin binds to the carboxyl-terminal end of helix 2B in the rod domain and phosphorylation of the HD interferes with the interaction between the two portions, determined using surface plasmon resonance techniques. These results, together with the reported IF model structures [7–10,32,33], pave the way for understanding molecular mechanisms of IF assembly and disassembly.

2. Materials and methods

2.1. Expression and purification of polypeptides

Full-length murine vimentin cDNA (1.4 kb) flanked by *Nde*I and *Bam*HI restriction sites was constructed by polymerase chain reaction (PCR) amplification using DNA from a mouse NIH3T3 library as template, cloned into pT7blue(R) (Novagene, Madison, WI, USA) and confirmed by DNA sequencing. DNA fragments coding the vimentin α-helical rod domain and subdomains (Fig. 1), flanked by *Nde*I and *Bam*HI restriction sites, were also constructed by PCR amplification using the vimentin cDNA as template and cloned as above. These were inserted into a *Nde*I–*Bam*HI-digested pET-3a plasmid (Novagene, Madison, WI, USA), and expressed in BL21(DE)-pLysS cells (Novagene, Madison, WI, USA). The expressed polypeptides in bacteria were purified by ion-exchange chromatography on Q- and SP-Sepharose resin (Pharmacia Biotech, Uppsala, Sweden). The amino-terminal HD was excised from the full-length vimentin with lysyl endopeptidase (Wako Pure Chemical, Osaka, Japan) and purified by SP-Sepharose chromatography. The authenticity of the poly-

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Abbreviations: IF, intermediate filament; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate

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peptides was verified by SDS–polyacrylamide gel electrophoresis and amino-terminal sequence analysis. Concentrations of the polypeptides were determined spectrophotometrically.

2.2. Phosphorylation of vimentin HD

Vimentin HD (8.2 μM) was phosphorylated by incubation with 5 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 1 mM ATP, 100 nM calyculin A, 2 mM $MgCl_2$, 25 mM Tris–HCl, pH 7.5, at 25°C for 1 h. Phosphorylated HD was isolated by the column chromatography.

2.3. Peptide synthesis

Peptide corresponding to the carboxyl-terminal 20 amino acid residues of helix 2B was synthesized by a solid-phase method using a 431A peptide synthesizer (Applied Biosystems, Foster City, CA, USA), purified by reversed-phase high-performance liquid chromatography, and the concentration was determined by amino acid analysis.

2.4. Binding affinity measurements

Interaction between polypeptides was determined using surface plasmon resonance technology on a BIAcore 1000 (BIAcore AB, Uppsala, Sweden). Since the HD has an α-amino group of the amino-terminal residue and an ε-amino group of carboxyl-terminal Lys-96, immobilization was carried out through these groups to the activated carboxyl groups of CM5 sensor chip, according to procedures of the manufacturer. Polypeptide helix 2B or the synthetic peptide was immobilized through the thiol group of the cysteine residue, with aid of a coupling reagent, 2-(2-pyridinyldithio)ethaneamine. Analytes were dissolved in 10 mM HEPES pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P-20, and were injected over the sensor chip at a flow rate of 10 μl/min at 25°C. Control experiments were performed on an unrelated protein (α-lactalbumin) surface. The blank sensorgram was subtracted from the assay curve, using BIA-evaluation 3.0 software. This software was used also to determine kinetic constants of the interaction.

3. Results and discussion

3.1. Preparation of polypeptides representing the vimentin domains and subdomains

To examine domain interactions involving the HD of vimentin, we expressed in *Escherichia coli* cDNAs encoding full-length mouse vimentin (465 amino acids) and its α-helical domain or subdomains, 1AB2AB (residues: 102–410), 1AB (102–244), 1B (150–244), L2AB (245–410) and 2B (314–410) (Fig. 1). After solubilization of cell lysates in 8 M urea, the polypeptides were purified by Q- and SP-Sepharose ion-exchange chromatography. The HD (residues: 1–96) was obtained by limited proteolysis of full-length vimentin with lysyl endopeptidase followed by SP-Sepharose chromatography.

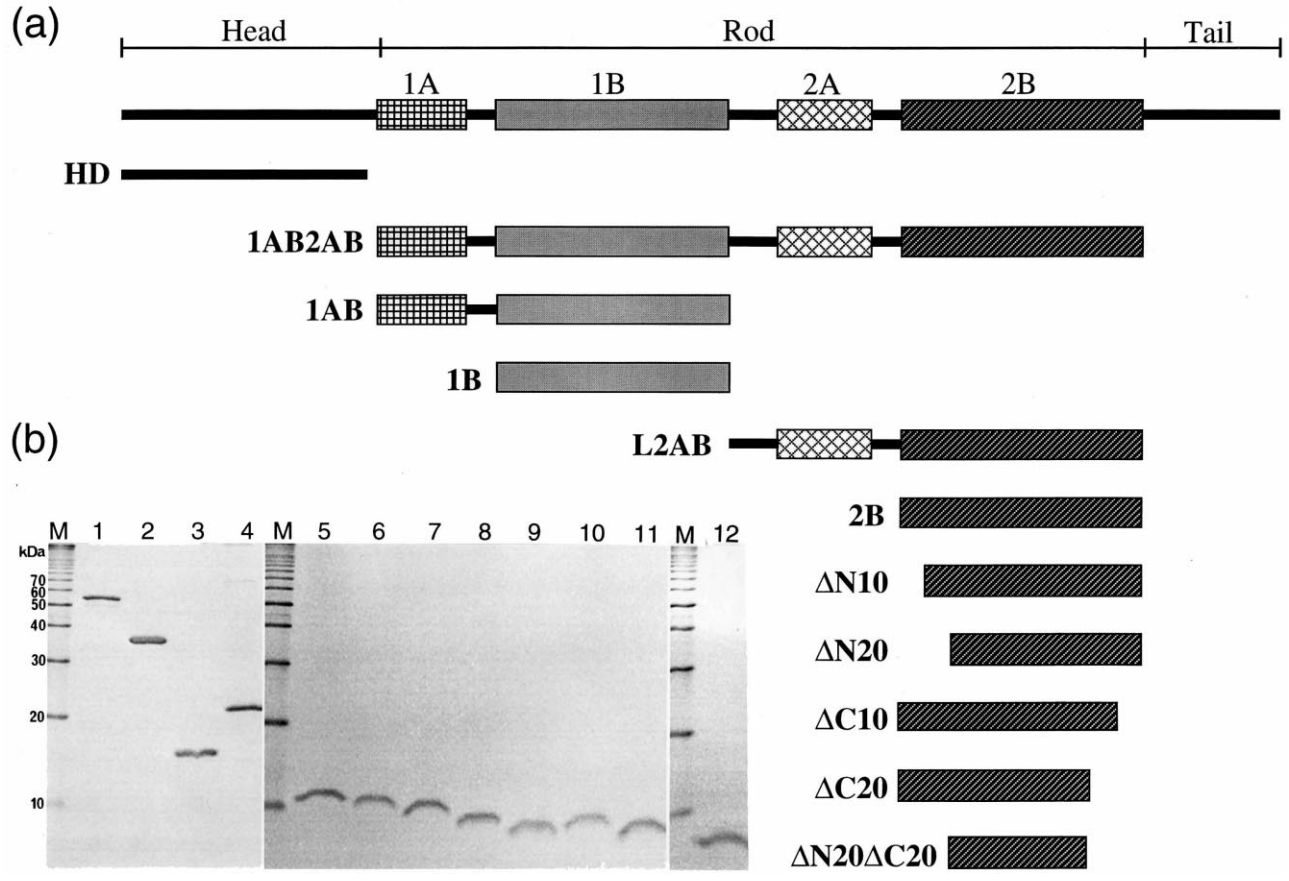

Fig. 1. Schematic presentation of the vimentin domains and subdomains used in this study (a), and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of the polypeptides (b). In (a), the boxes indicate the α-helices 1A, 1B, 2A and 2B. HD (amino acid residues in mouse vimentin: 1–96), the amino-terminal HD. 1B (150–244), the helix 1B. 1AB (102–244), the amino-terminal half of the rod domain and it contains the helices 1A and 1B. 2B (314–410), the helix 2B. L2AB (245–410), the carboxyl-terminal half of the rod domain and it contains the helices 2A and 2B. 1AB2AB (102–410), the rod domain containing the four helices. The deleted fragments of 2B are abbreviated as ΔNX or ΔCX, where ΔN stands for amino-terminal, and ΔC for carboxyl-terminal deletion, whereas X indicates the number of amino acids deleted. ΔN20ΔC20 lacks both the amino- and carboxyl-terminal 20 amino acid residues of 2B. In (b), purified polypeptides were resolved on a 17.5% polyacrylamide gel, stained with Coomassie blue: lane 1, full-length vimentin; lane 2, 1AB2AB; lane 3, 1AB; lane 4, L2AB; lane 5, HD; lane 6, 1B; lane 7, 2B; lane 8, ΔN10; lane 9, ΔN20; lane 10, ΔC10; lane 11, ΔC20; lane 12, ΔN20ΔC20.

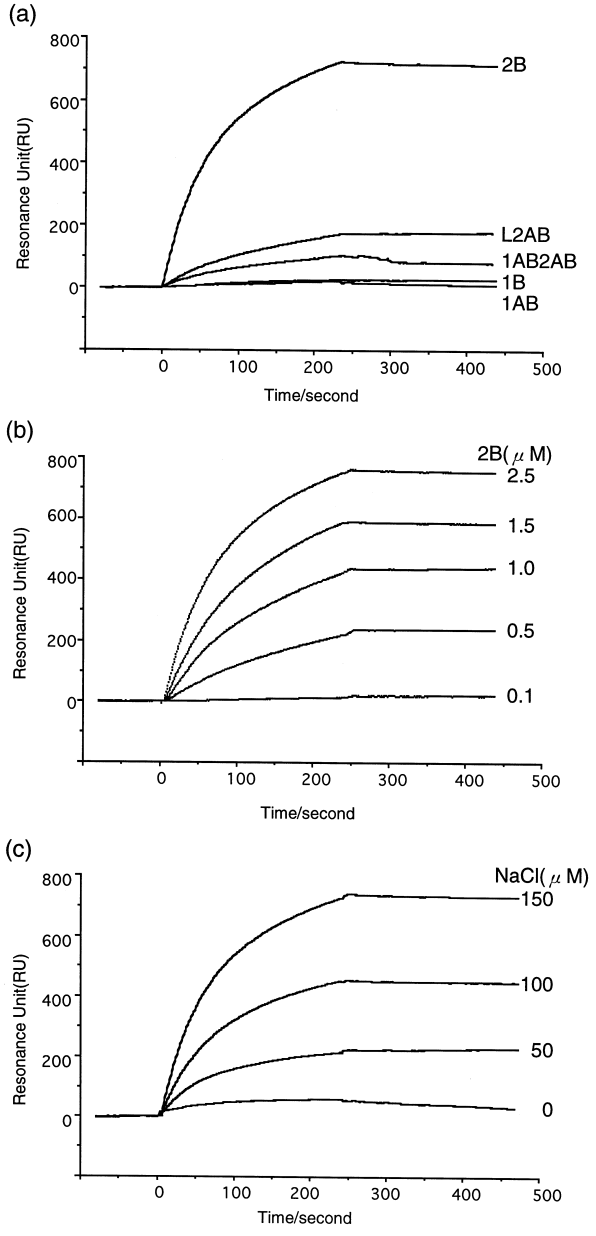

Fig. 2. Binding sensorgrams of polypeptides to the immobilized HD. HD was immobilized (4.5 ng/mm^2) on a dextran surface, as described under Section 2. Specific binding of each polypeptide to the immobilized HD was obtained by subtracting the blank sensorgram from the assay curve, as described under Section 2. (a) Polypeptides (2.5 μM) containing α-helical regions were injected. (b) Effect of the concentration (0.1–2.5 μM) of 2B on the interaction with the immobilized HD. (c) Effect of NaCl concentration (0–150 mM) on the HD–2B interaction.

Since the tail domain is not critical for IF formation [15–17], this domain was precluded as a candidate for a binding site of the HD.

Recombinant vimentin formed normal IFs in a standard assembly condition (10 mM imidazole–HCl containing 150 mM NaCl, pH 7.0). Circular dichroism curves of polypeptides 1AB, 1B, L2AB, 2B and 1AB2AB in 10 mM HEPES–NaOH containing 150 mM NaCl, pH 7.5 indicated that they adopt α-helical conformation (data not shown).

3.2. Interaction of the α-helical domain or subdomains with the immobilized HD

Interactions between HD and the α-helical polypeptides were examined using surface plasmon resonance. At first, HD was immobilized as a ligand, on a dextran surface of a sensor chip. When the polypeptides 1AB, 1B, L2AB, 2B and 1AB2AB as analytes at a concentration of 2.5 μM were injected over the HD-immobilized sensor chip, we obtained the binding sensorgrams shown in Fig. 2a. While 1AB or 1B (0.1–20 μM) showed no detectable binding, polypeptides such as 2B, L2AB and 1AB2AB interacted with the immobilized HD. Of these polypeptides, 2B exhibited the highest binding level. This binding depended on the concentration of 2B (Fig. 2b) and required the presence of 150 mM NaCl (Fig. 2c). Since IF assembly from vimentin tetramers occurs under the physiological ionic strength [11], the requirement of salt was reasonable as an interaction which associates tetramers into IF. These results suggest that the HD of vimentin interacts with the helix 2B during IF formation.

3.3. Effect of phosphorylation on the interaction between the HD and the immobilized helix 2B

To confirm the interaction between HD and 2B, 2B was immobilized on a dextran surface through the thiol group of the cysteine residue (Cys-327). When HD (0.6–10 μM) was injected as an analyte, it bound to the immobilized 2B (Fig.

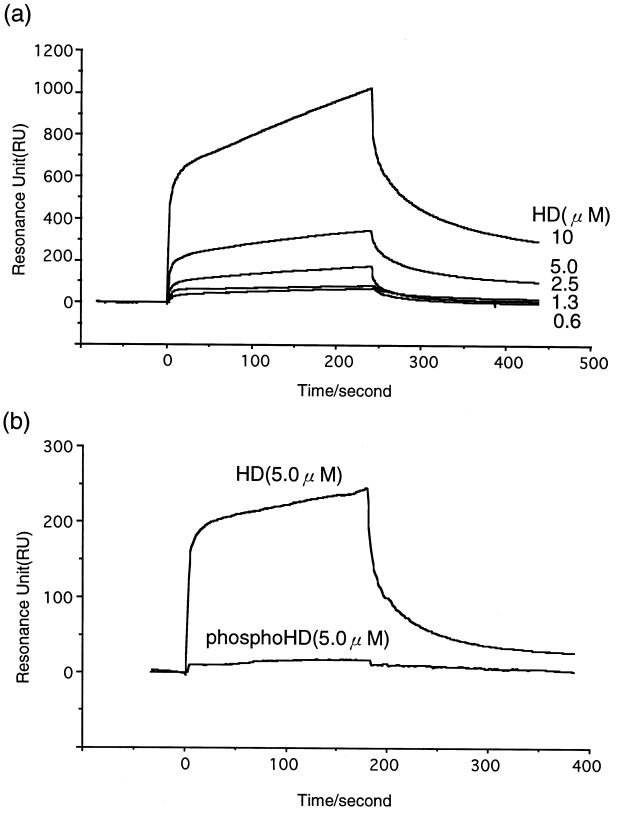

Fig. 3. Binding sensorgrams of HD or phosphorylated HD to the immobilized 2B. 2B was immobilized (0.152 ng/mm^2) on a dextran surface, as described under Section 2. (a) Effect of the concentration (0.6–10 μM) of HD on the interaction with the immobilized 2B. (b) Effect of phosphorylation of HD on the interaction with 2B. HD phosphorylated, as described under Section 2 was injected at the concentration of 5 μM. Compared to HD, the phosphorylated HD apparently lost potential to interact with 2B.

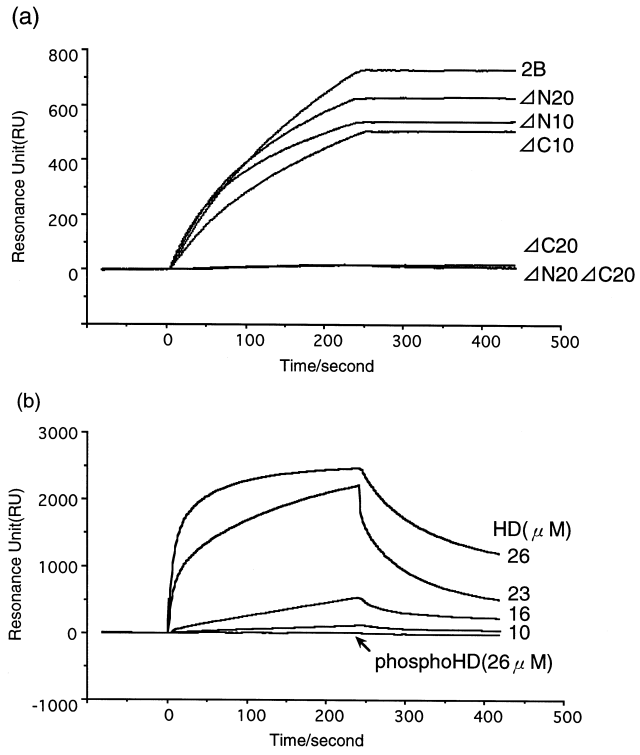

Fig. 4. Binding sensorgrams of amino- and/or carboxyl-terminal deleted 2B fragments (2.5 μM) to the immobilized HD (a), and binding sensorgrams of HD (10–26 μM) and phosphorylated HD (26 μM) to the immobilized synthetic peptide representing the carboxyl-terminal 20 amino acids of 2B. In (b), the synthetic peptide was immobilized (0.19 ng/mm^2) on a dextran surface, as described under Section 2.

3a). Kinetic analysis showed that the K_d value for the interaction is 1.4 μM. Since phosphorylation of the HD results in disassembly of vimentin IF in vivo or in vitro [21–31], it was of interest to examine the effect of phosphorylation on the HD–2B interaction. HD was phosphorylated with cAMP-dependent protein kinase, and approximately 3 mol of phosphates were incorporated into the HD molecule. When the phosphorylated HD (2.5 or 5 μM) was injected over the immobilized 2B, no interaction was observed (Fig. 3b). Thus, phosphorylation inhibited the HD–2B interaction.

3.4. Localization of HD-binding site in the helix 2B

To localize the HD-binding site in 2B, amino- and/or carboxyl-terminally truncated forms of 2B (Fig. 1) were expressed in bacteria and purified. ΔN10 and ΔN20 lack the amino-terminal 10 and 20 amino acid residues of 2B, respectively. ΔC10 and ΔC20 lack the carboxyl-terminal 10 and 20 amino acid residues, respectively. ΔN20ΔC20 lacks both the amino- and carboxyl-terminal 20 amino acid residues. Of these polypeptides, ΔC20 and ΔN20ΔC20 (2.5–20 μM) failed to bind with the immobilized HD (Fig. 4a). Removal of the amino-terminal 20 or the carboxyl-terminal 10 amino acid residues of 2B had no significant effect on the interaction. In addition, we observed that the immobilized synthetic peptide (sequence: CALDIEIATYRKLLEGEESRI), which corresponds to the carboxyl-terminal 20 amino acid residues of 2B, has a substantial affinity with HD but not with the phosphorylated one (Fig. 4b). These results suggest that the carboxyl-terminal 20 amino acids of helix 2B represent a binding site for the HD and that the interaction is interfered with by phosphorylation of the HD.

3.5. Conclusion

At low ionic strength, IF subunit proteins form coiled-coil dimers with the two chains arranged in parallel and in register [7–10]. Dimers readily associate to form stable tetramers in which dimers are antiparallel, and are either arranged in register or are staggered [7–10,32,33]. While at physiological ionic strength, tetramers are supposed to align laterally and longitudinally to form IF, molecular mechanisms underlying the polymerization steps have remained obscure. In the IF structures, HDs of a tetramer would have to face the amino- and carboxyl-terminal ends of the rod domains of another tetramer. Both ends of the rod domains show a high degree of sequence conservation, and deletion or mutations in the regions of IF proteins perturb or abolish polymerization, indicating that both ends of the rod domains play a role in IF assembly [7–10]. HDs, which vary in size and sequence but commonly have a wealth of arginine residues, are also crucial, since IF proteins lacking the HD or having the phosphorylated one remain in tetramers and are assembly incompetent [3,15,18–31]. The results obtained here emphasize the significance of the interaction between the HD and the carboxyl-terminal end of the rod domain in vimentin IF formation. Traub et al. reported the salt-stable interaction of the HD of vimentin with the carboxyl-terminal half of the rod domain rather than the amino-terminal half of the domain [34]. Our results suggest that the carboxyl-terminal 20 amino acids of helix 2B in the rod domain function as an acceptor site for the HD in vimentin IF formation, and that the interaction is controlled by the HD-specific phosphorylation.

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