Characterization of the Nucleic Acid Binding Region of the Intermediate Filament Protein Vimentin by Fluorescence Polarization

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ABSTRACT: Employing deletion mutant proteins and fluorescein-labeled oligodeoxyribonucleotides in a fluorescence polarization assay, the nucleic acid binding site of the intermediate filament (IF) subunit protein vimentin was localized to the middle of the arginine-rich, non-α-helical, N-terminal head domain. While deletion of the first few N-terminal residues (up to amino acid 17) had almost no effect, deletions of residues 25−64 or 25−68 essentially abolished the binding of nucleic acids by the respective proteins. Proteins with smaller deletions, of residues 25−39 or 43−68, were still able to bind nucleic acids quite well at low ionic strength, but only the proteins containing the first DNA-binding wing (residues 27−39) retained the ability to stably bind nucleic acids at physiological ionic strength. These results were confirmed by data obtained with two synthetic peptides whose sequences correspond to the smaller deletions. Nitration experiments showed that one or more of the tyrosines in the head domain are responsible for the stable binding by intercalation. Interestingly, the residues responsible for binding nucleic acids can be deleted without major influence on the in vivo polymerization properties of the mutant proteins. Only the protein with the largest internal deletion, of residues 25−68, failed to form filaments in vivo. Since the N-terminal head domains of IF proteins are largely exposed on the filament surface, but nevertheless essential for filament assembly, these results support the model that the middle of the head domain of vimentin may loop out from the filament surface and thus be available for interactions with other cellular structures or molecules.

It has been known for many years that the nonepithelial cytoplasmic intermediate filament (IF)[1] subunit proteins bind quite well to a variety of nucleic acids (*1* and references therein). This property has been the root of much unresolved controversy in the field, with most investigators adopting a "wait and see" attitude toward the biological relevance of this behavior, especially since the cytoplasmic IF proteins do not possess nuclear localization signals and are not known to be present in the nucleus. Nonetheless, based on a plethora of activities of cytoplasmic IF proteins and comparisons to gene regulatory and karyoskeletal proteins, we have proposed that cells might indeed exploit the nucleic acid-binding property of IF proteins in global regulation of gene expression and other DNA-based activities such as recombination and repair (*2*), i.e., in events that normally take place within the confines of the nucleus. Considerable support for this hypothesis has been provided by the recent demonstration that a variety of nucleic acids, from oligonucleotides to certain linear double-stranded DNAs to superhelical plasmids and the replicative form of the simian virus 40 genome, are capable of transporting the IF subunit protein vimentin (*3*) as well as other substances (*4*) into the nucleus in vivo. This transport was observed after microinjection of complexes formed in vitro and even after microinjection of the nucleic acid alone into cells containing labeled vimentin incorporated into IFs (*3*), demonstrating that the transport of vimentin into the nucleus can indeed occur under in vivo conditions. In these experiments, it was noted that only those nucleic acids to which vimentin bound well in vitro were capable of transporting it into the nucleus in vivo. Furthermore, vimentin incorporated into the nucleus of mouse or human skin fibroblasts could not be detected immunologically but was readily detected when directly labeled with various fluorophores or biotin. This suggested that the immunologically reactive epitopes of the newly transported, intranuclear vimentin were masked in vivo by either their interaction with the nucleic acid transporter or by unidentified nuclear components with which vimentin might interact. In this light, it is worth repeating that vimentin is known to bind strongly not only to certain, mostly repetitive and mobile genomic DNA sequences (*5*; Tolstonog, G., and Traub, P., unpublished results), but also to ribosomal and heterogeneous nuclear RNA and core histones (*6*, *7*; see refs *2*, and *8* for discussion and further references). While previous experiments have investigated the specificity, kinetics, and stoichiometry of nucleic acid binding by vimentin in detail (*16*, *19*, *1* and references therein) and have demonstrated that the nucleic acid-binding region of vimentin is localized to the non-α-helical head domain, the exact residues contributing to this binding have not been identified experimentally. We, therefore, elected to use a fluorescence polarization assay (*15*) to investigate which amino acid residues in the vimentin head domain contribute to the binding of nucleic acids. Three

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[1] Abbreviations: IF, intermediate filament; F-ODN, fluorescein-labeled oligodeoxyribonucleotide; DAPI, 4,6,diamidino-2-phenylindole; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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Table 1: Amino Acid Sequences of the Wild Type and Deletion Vimentin Proteins and Proteolytic Fragments[a]

```
mouse vimentin
          .10         .20         .30         .40         .50         .60         .70         .80         .90
STRSVSSSSY.RRMFGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

T-vimentin:
----------.----------.----------.----------.----------.----------.----------.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NT1:
STRSVSSSSY.RRMFGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK

NΔ10:
----------.RRMFGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ13:
----------.---FGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ17:
----------.-------GTS.SRPSSNRSYV.TTSTRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ32:
----------.----------.----------.--STRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ25-68:
STRSVSSSSY.RRMFGGSGTS.SRPS------.----------.----------.----------.--------LR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ25-63:
STRSVSSSSY.RRMFGGSGTS.SRPS------.----------.----------.----------.---SSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ25-38:
STRSVSSSSY.RRMFGGSGTS.SRPS------.--------LG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

NΔ43-68:
STRSVSSSSY.RRMFGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SA--------.----------.---------LR.SSVPGVRLLQ.DSVDFSLADA.INTEFK***
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[a] Missing residues are indicated by a -. The symbols **** are used to indicate that the rest of the sequence is identical to that of wild type vimentin. The numbers following the "." above the mouse vimentin sequence indicate the number of residues preceding each ".".

Table 2: The Sequence of Two Synthetic Peptides (bold) is Indicated within the Wild Type Vimentin Sequence (light gray)[a]

```
mouse vimentin
          .10         .20         .30         .40         .50         .60         .70         .80         .90
STRSVSSSSY.RRMFGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SALRPSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

R23R (residues 22-44):
STRSVSSSSY.RRMFGGSGTS.SR[RPSSNRSYV.TTSTRTYSLG.SALR]PSTSRS.LYSSSPGGAY.VTRSSAVRLR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****

R25R (residues 44-68):
STRSVSSSSY.RRMFGGSGTS.SRPSSNRSYV.TTSTRTYSLG.SALR[PSTSRS.LYSSSPGGAY.VTRSSAVR]LR.SSVPGVRLLQ.DSVDFSLADA.INTEFK****
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[a] The numbers following the "." above the mouse vimentin sequence indicate the number of residues preceding each ".".

approaches were taken to address this question. In the first, specific stretches of amino acids throughout the head domain were deleted via recombinant DNA technology, producing a battery of recombinant proteins (Table 1). In the second approach, the contribution of the five tyrosine residues in the head domain was evaluated by their chemical modification (nitration). In the third approach, smaller synthetic peptides corresponding to distinct regions of the N-terminal head domain were evaluated for their ability to bind nucleic acids. We also investigated the in vivo polymerization properties of several vimentin deletion proteins to see whether specific regions important for nucleic acid binding are dispensable for IF formation.

EXPERIMENTAL PROCEDURES

Peptides and Proteins. Wild-type mouse vimentin was prepared from Ehrlich ascites tumor cells as previously described (*9*). T-vimentin, the amino terminal peptide NT1 (Table 1) and nitrated NT1 were prepared as described (*1, 10*). Synthetic peptides R23R (M_W 2561) and R25R (M_W 2630), which are identical in sequence to amino acid residues 22−44 and 44−68, respectively, of the amino terminal head domain of mouse vimentin (Table 2), were purchased from either Neosystems (Strasbourg, France) or Eurogentec (Seraing, Belgium). Recombinant mouse vimentin and vimentin deletion proteins were synthesized in *Escherichia coli* via standard techniques of recombinant DNA technology and purified as described (*11*). All of the recombinant proteins employed in this study are identical to the wild-type mouse vimentin sequence, minus the indicated deletions (i.e., there are no nonvimentin residues at the deletion junctions)(see Table 1). In this paper, amino acid residues are numbered from the first serine (see Table 1), which is the authentic N-terminal residue of the wild-type mouse vimentin. The proteins were typically >90% pure (based on SDS-gel electrophoresis) and possessed the expected molecular weights, as determined by MALDI-TOF mass spectrometry, using a Kratos MALDI III instrument (Shimadzu, Duisburg, Germany) in linear, high mass mode and standard software (Kratos Kompact manual and software version 5.2). Since

no differences were observed in these assays between authentic mouse vimentin (i.e., isolated from Ehrlich ascites tumor cells) or NT1 and recombinant protein produced from the mouse vimentin coding sequence, the name vimentin will be used throughout this manuscript to describe the protein derived from either source. Protein concentrations were determined with the BioRad Protein Assay (BioRad, Munich, Germany) using bovine serum albumin as a standard. Alternatively, the concentration of NT1 in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, was determined spectrophotometrically, using a ϵ_{274nm} of 0.545 OD mg^{-1} or a ϵ_{280nm} of 0.478 OD mg^{-1}.

Nucleic Acids. Unless otherwise noted, all nucleic acid manipulations were as described in standard protocols (*12*, *13*) or in previous publications (*14*, *15*). Nucleic acid concentrations were determined spectrophotometrically, using a constant factor of 1 mg/mL = 20 OD_{260nm} for all nucleic acids (*16*). Oligonucleotides labeled with a single fluorescein at their 3′ ends (F-ODNs) were prepared as previously described (*14*, *15*) and were routinely stored in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, at 1−5 mg ml^{-1} at 4 °C. Oligonucleotide stock solutions were briefly boiled and rapidly cooled in ice to disrupt inter- and intramolecular complexes prior to dilution into the reaction buffer.

Fluorescence Polarization Assay. Nucleic acid/protein or peptide complex formation was monitored by fluorescence polarization as described using a Beacon fluorescence polarization system (Pan Vera Corp., Madison, WI) (*15*) for single cuvettes or a Polarion microtiter plate fluorescence polarization system (Tecan Austria GmbH, Grödig/Salzburg, Austria). Both systems were used according to the manufacturer's instructions and with standard filters for fluorescein detection (Polarion: excitation 485 nm, emission 535 nm), using 12 mm glass test tubes for the Beacon system (1 mL final reaction volume) or Greiner 96-well, flat bottom plates (black polystyrene, medium binding capacity, #655076; Greiner Labortechnik, Frickenhausen, Germany) for smaller reaction volumes (typically 200 μl). All reactions contained 10 nM F-ODN and varying concentrations (see Figures) of the proteins or peptides in a low ionic strength buffer (to prevent polymerization of the subunit proteins into IFs) consisting of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, without added KCl (unless otherwise indicated). For some measurements of the synthetic peptides, custom 96-well, flat bottom, black polypropylene plates (Greiner) were also used. Individual data points for the Polarion are the averages of 100 measurements/well. All results presented in the figures are the means (with standard deviations indicated) of three to eight independent replicates for each sample and were measured with the Polarion. A 1 nM sodium fluorescein stock solution was prepared from a 10% fluorescein solution (Sigma, Deisenhofen, Germany) in 10 mM $NaHCO_3$, pH 9, using a molar extinction coefficient of ϵ_{492nm} = 77 000 (Molecular Probes, Eugene, OR) and was used to calibrate the machines. Measurements were made at 37 °C, although qualitatively identical results were also obtained at room temperature with the Polarion. Nucleic acid/protein or peptide complex stability was estimated by remeasurement of the samples after the addition of small volumes of a concentrated KCl solution (1−4 M, depending on the final [KCl] desired) to calibration standards, blanks and experimental samples. Complexes were presumed to have dissociated when their fluorescence polarization values fell to that of the oligonucleotide alone. Fluorescence polarization values are the ratios of the fluorescence measured in two planes through polarizing filters and are, therefore, dimensionless; to avoid the unnecessary repetitive printing of values to 3 decimal places, they are expressed as millipolarization (mP) (i.e., a fluorescence polarization value of 0.277 is expressed as 277 mP). Since important parameters (viscosity, temperature, fluorophore, and complex type) were kept constant and fluorescence intensities remained relatively constant over the concentrations of ligands employed, the changes in fluorescence polarization observed are directly proportional to changes in the molecular weight of the complexes (see ref *17* for details and further references).

Cell Culture, Microinjection, and Confocal Laser Scanning Microscopy. Human adenocarcinoma SW 13 cells, deficient in endogenous cytoplasmic IF proteins (subclone F8) or simian virus 40-transformed fibroblasts derived from vimentin knockout mouse embryos (obtained from Dr. R. Evans, University of Colorado Health Sciences Center, Denver, CO), were cultured and microinjected with the vimentin proteins as previously described (*3*, *18*). Cells were fixed and processed for immunomicroscopy to detect vimentin proteins at 4 or 24 h after microinjection as previously described (*3*). Cell nuclei were also stained with DAPI according to standard protocols. Confocal laser scanning microscopy and image processing were as previously described (*14*), except that a Leica TCS NT microscopy system (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) was used and the fluorescence signal from the DAPI channel was pseudocolored blue during image processing.

Other Procedures and Reagents. Fluorescence polarization data were transferred to and graphs were prepared with SigmaPlot for Windows Version 4.01 software (SPSS ASC GmbH, Erkrath, Germany). Other reagents were as specified in the references or were of reagent grade from Sigma or Merck (Darmstadt, Germany).

RESULTS

As in previous studies with mouse vimentin isolated from Ehrlich ascites tumor cells (*15*), recombinant mouse vimentin showed a pronounced base composition specificity for binding of F-ODNs. For example, as seen in Figure 1, F−d$(A)_{25}$ was not bound at any concentration tested, whereas F−d$(G)_{25}$ was bound quite well by intact recombinant mouse vimentin, but neither F-ODN was bound by T-vimentin, which lacks the first 70 amino acids (Table 1). In additional experiments with recombinant mouse vimentin in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, a K_d of ca. (0.8−1) × 10^{-8} M was determined by Klotz plot analysis for the binding of F−d$(G)_{25}$ (data not shown). This compares favorably with the K_a of 4 × 10^7 M^{-1} determined by a filter binding assay for the binding by vimentin of a G-rich telomere model oligonucleotide with single-stranded overhangs (*16*). Since vimentin binds dG homopolymers somewhat better than the telomere oligonucleotides (*16*), it is apparent that the fluorescence polarization assay yields similar results to those previously obtained with the classical filter binding assay. As shown in Figure 1, NT1 (see Table 1) bound F−d$(G)_{25}$ in a manner essentially indistinguishable from intact vimentin.

As can be seen in Table 1, a variety of vimentin deletion mutant proteins were prepared that were lacking various

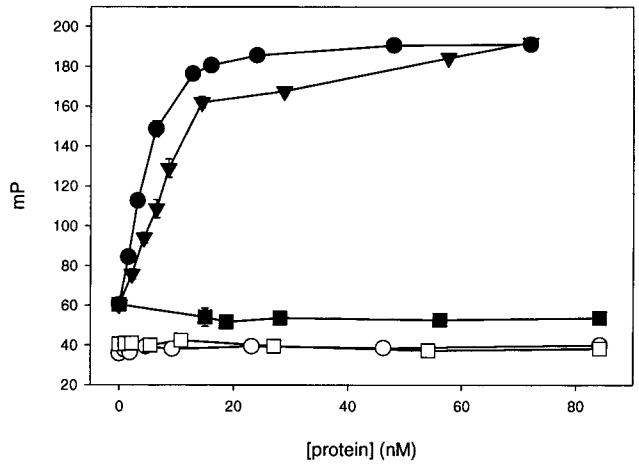

FIGURE 1: Binding of F-oligonucleotides by recombinant mouse vimentin, the N-terminal head domain peptide NT1 and T-vimentin in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5. F−d(A)$_{25}$ (open symbols) and F−d(G)$_{25}$ (filled symbols) were incubated with increasing amounts of vimentin (circles), NT1 (triangles), and T-vimentin (squares) as described in Experimental Procedures for 15 min at 37 °C. Fluorescence polarization is expressed as mP (see Experimental Procedures).

amino acids of the non-α-helical head domain. Each was tested, along with T-vimentin, for its ability to bind to F−d(G)$_{25}$, i.e., an F-ODN to which full-length vimentin binds quite well. As found previously with other nucleic acids, T-vimentin was unable to bind F−d(G)$_{25}$, F−d(A)$_{25}$ (Figure 1), or any other F-ODN tested (data not shown). Removal of the first 10 or 13 residues from the N-terminus of vimentin had essentially no effect on the ability of the modified proteins to bind oligonucleotides (Figure 2A). Removal of several more residues, i.e., up to 17 or 32 (Table 2), yielded proteins that were still able to interact with F−d(G)$_{25}$ quite well in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (Figure 2A), albeit reduced compared to full-length vimentin, but which showed greatly reduced affinities at higher ionic strengths (data not shown). Thus, the amino acid residues contributing to the binding of oligonucleotides must lie between residues 14 and 69.

Internal deletions of residues 25−63 resulted in a protein with a reduced capacity to bind to F−d(G)$_{25}$ (Figure 2B). Deletion of five additional residues, including arginine 68, essentially abolished the ability of the modified protein to bind oligonucleotides (Figure 2B). The binding of F−d(G)$_{25}$ by the NΔ25−63 and NΔ25−68 proteins could be abolished by KCl added to >25 mM (data not shown). Smaller internal deletions NΔ25−38 and NΔ43−68, which roughly correspond to the first and putative second DNA-binding ladder of vimentin (see ref *1* and Discussion), gave rise to mutant proteins with considerable capacity to bind F−d(G)$_{25}$ (Figure 2B) and with only a slightly reduced affinity (about 2−3-fold) relative to intact vimentin. The oligonucleotide/protein complexes formed by the NΔ25−38 protein were sensitive to dissociation by added KCl in excess of 50 mM (data not shown), whereas those formed by the NΔ43−68 protein were essentially resistant to dissociation by KCl at 150 mM (Figure 2B).

The purified N-terminal head domain of vimentin, NT1, with its 5 tyrosine residues nitrated, bound F−d(G)$_{25}$ in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, in a manner identical to that of the unmodified NT1 peptide (Figure 3A). However,

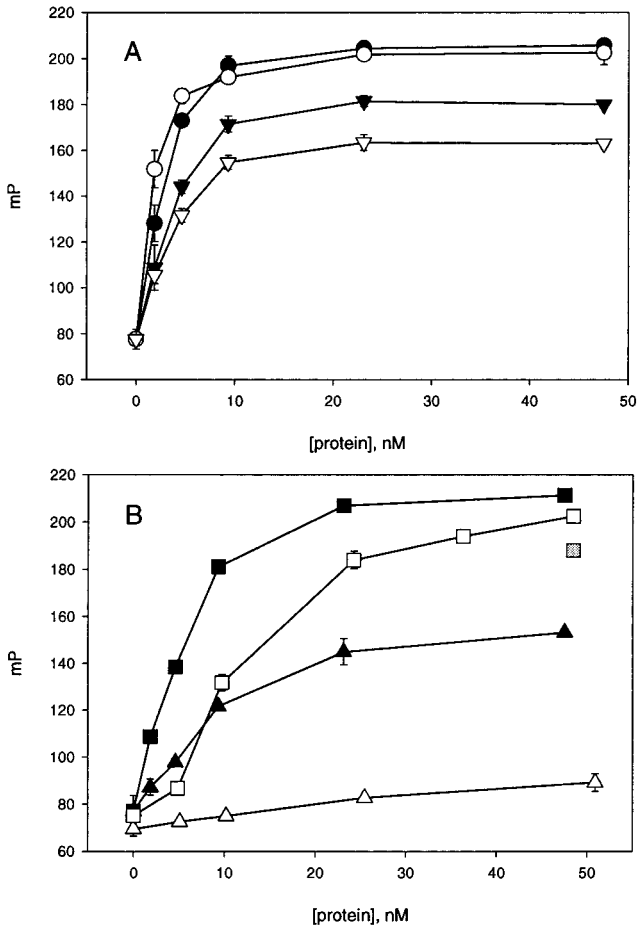

FIGURE 2: Binding of F−d(G)$_{25}$ by various vimentin deletion mutant proteins in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5. (A) Influence of N-terminal sequences on the binding. Proteins lacking the N-terminal 10 (NΔ10, filled circles), 13 (NΔ13, open circles), 17 (NΔ17, filled triangles) and 32 (NΔ32, open triangles) amino acid residues were tested for their ability to bind F−d(G)$_{25}$. (B) Influence of internal deletions on the binding. The proteins NΔ25−38 (filled squares), NΔ43−68 (open squares), NΔ25−63 (filled triangles) and NΔ25−68 (open triangles) were tested for their ability to bind F−d(G)$_{25}$. The gray square represents the binding of F−d(G)$_{25}$ by the NΔ43−68 protein after the addition of KCl to 150 mM.

the complexes formed by the nitrated peptide were completely dissociated by 50 mM KCl, whereas those formed by the unmodified peptide were largely unaffected by salt up to 150 mM (Figure 3B); their salt resistance was similar to that of the complexes formed by vimentin (Figure 3B).

Since the synthetic peptides R23R and R25R bound to the polystyrene microtiter plates, it was necessary to measure them in glass cuvettes in the Beacon system (data not shown) or in black polypropylene microtiter plates in the Polarion (Figure 4). The curves obtained for the binding of F−d(G)$_{25}$ at low ionic strength for both R23R (which corresponds to the first DNA-binding wing) and R25R (the putative second DNA-binding wing) were very similar (Figure 4). The binding affinities of these peptides are about 20-fold lower than those exhibited by the intact N-terminal head domain, NT1, or full-length vimentin. The binding of F−d(G)$_{25}$ by R23R was largely resistant to the addition of KCl to 150 mM, whereas the binding by R25R was largely abolished by the addition of KCl to 150 mM (Figure 4, filled symbols for 1000 nM peptide and data not shown).

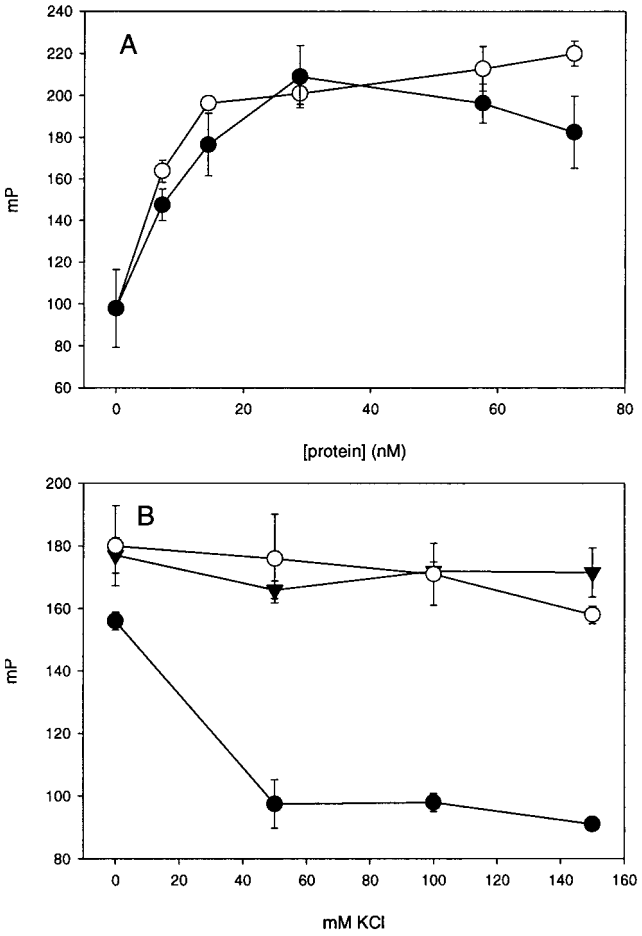

FIGURE 3: Binding of F−d(G)$_{25}$ by NT1 and nitrated NT1. In 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (A), both NT1 (open circles) and nitrated NT1 (filled circles) bound the F−d(G)$_{25}$ in a manner very similar to that of the wild-type, full-length protein. At higher ionic strengths (B), the binding of F−d(G)$_{25}$ by both NT1 (open circles) and vimentin (filled triangles) was relatively stable and essentially identical. However, the complexes formed by the nitrated NT1 (filled circles) were more labile and were completely dissociated by KCl added to 50 mM (or higher concentrations).

The ability of some of the vimentin deletion mutant proteins to polymerize in vivo was tested by their microinjection into SW 13 cells (Figure 5) or simian virus 40-transformed fibroblasts derived from vimentin knockout mouse embryos (data not shown). Cells were fixed and processed for confocal laser scanning microscopy, using standard procedures either 4 (data not shown) or 24 h after microinjection. As in other experiments, the wild-type, full-length vimentin polymerized into filaments and bundles of filaments that appear to be identical to authentic vimentin IFs (Figure 5A). Both the NΔ25−38 and the NΔ43−68 proteins also polymerized into filaments that were indistinguishable from those formed by full-length vimentin (Figure 5B, C). The NΔ25−63 protein formed filamentous structures (Figure 5D) that appeared shorter and thinner than those formed by the other proteins. However, the NΔ25−68 protein failed to form any type of filamentous structures and produced only nonfilamentous aggregates or diffuse staining in the cytoplasm after 4 h (data not shown) or after 24 h (Figure 5E). A summary of the DNA-binding and polymerization properties of the proteins and peptides tested is presented in Table 3.

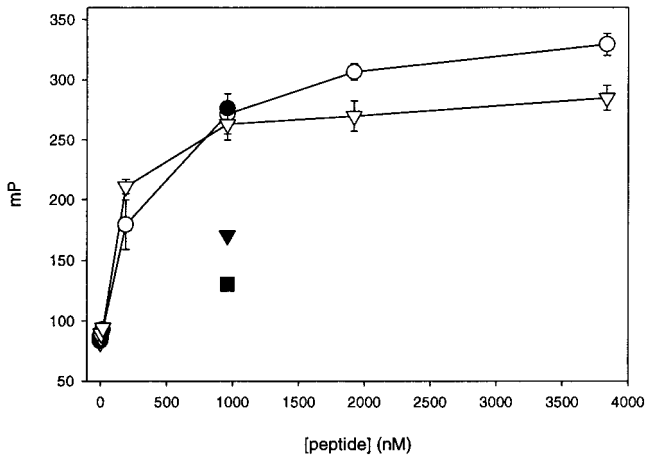

FIGURE 4: Binding of F−d(G)$_{25}$ by synthetic peptides of the N-terminal head domain of vimentin. R23R (circles) and R25R (triangles) were tested for their ability to bind F−d(G)$_{25}$ in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (open symbols), and after the addition of KCl to 150 mM (closed symbols, data only shown for 1000 nM peptide). While both peptides bound the F−d(G)$_{25}$ at low ionic strength, the binding by R23R was relatively resistant to salt, whereas that by R25R was not. The blank value for the F−d(G)$_{25}$ alone at 150 mM KCl (filled square) is much higher than at low ionic strength due to the formation of G-tetrads and other higher order structures.

DISCUSSION

Since all of the wild-type and mutant vimentin proteins listed in Table 1 have intact rod domains and are, in fact, identical in sequence following residue 68 (70 for T-vimentin), they should all be present as tetramers under the low ionic strength conditions (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) employed for the majority of these assays. Therefore, the stoichiometry of binding for these proteins should be similar to that observed for intact mouse vimentin under these conditions (i.e., 1.2 mole oligonucleotide per mole of protein tetramer)(*19*), although this was not measured in the present study. The assay employed here permits a relative comparison of the binding properties of the vimentin proteins (Table 1), the unmodified and nitrated NT1 peptides and the two synthetic peptides R23R and R25R (Table 2) among themselves; however, the results obtained with the peptides cannot be directly compared with those obtained with the proteins, since nothing is known about their polymerization status or stoichiometry in binding to nucleic acids. Nonetheless, the results presented in this study clearly demonstrate that the region of vimentin responsible for nucleic acid binding resides within the first 70 amino acid residues, since T-vimentin (produced by cleavage with thrombin) is completely inactive in binding. As can be seen in Table 1, the N-terminal head domain of vimentin (roughly the first 100 residues) contains 6 aromatic residues (5 tyrosines, 1 phenylalanine), 14 basic residues (13 arginines, 1 lysine) and 4 acidic residues (3 aspartic acids, 1 glutamic acid). Since all of the acidic residues, 2 of the arginines and the lysine residue occur in a cluster at the carboxyl end of the head domain (between residues 77 and 98), it is clear that they do not play a role in nucleic acid binding. Likewise, the first 3 arginines (at positions 3, 11, and 12) and the first tyrosine do not seem to play an important role in nucleic acid binding, since the NΔ10 and NΔ13 mutant proteins bind nucleic acids in a fashion essentially identical to the full-

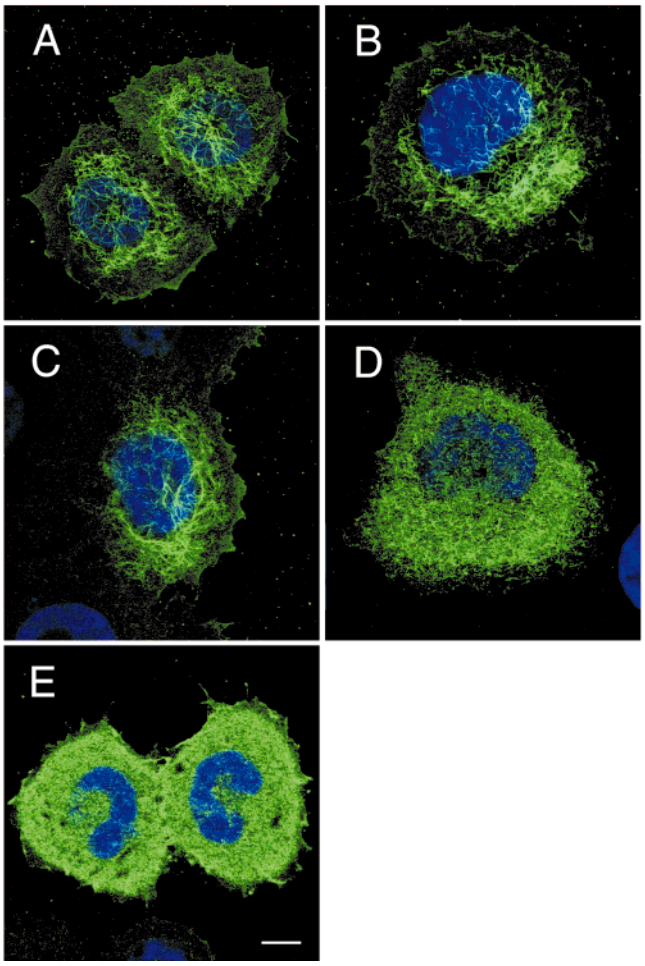

FIGURE 5: Confocal laser scanning microscopy of human adenocarcinoma SW13 cells deficient in endogenous cytoplasmic IF proteins after microinjection with various vimentin proteins and reaction with a goat anti-vimentin antibody and an FITC-labeled anti-goat secondary antibody (green pseudo-color) and counterstaining with DAPI (blue pseudo-color). Cells were microinjected with (A) full-length vimentin, (B) NΔ25−38, (C) NΔ43−68, (D) NΔ25−63, and (E) NΔ25−68 proteins. The cells were fixed and processed approximately 24 h after microinjection of the proteins. Each panel is a single confocal section chosen to show the typical structures observed with each protein. Bar, 10 μm.

length protein (Figure 2). Deletion of the middle of the head domain (i.e., NΔ25−68, Figure 2) essentially abolished the ability of the resulting protein to associate with nucleic acids.

Previous spectroscopic experiments have shown that 1 or more tyrosine residues in the N-terminal head domain of vimentin change from a polar to a less polar environment upon binding to DNA, indicating an intercalation or aromatic ring stacking interaction (*1*). In the present experiments, we have shown that the nitration of the tyrosines of the N-terminal peptide NT1 has only little effect on the electrostatic binding of oligonucleotides, but essentially abolishes the salt-stable, presumably intercalation, interactions (Figure 3).

These data strongly implicate that the 7 arginine and 4 tyrosine residues within the middle of the head domain (i.e., from residues 20−68) are responsible for the electrostatic and intercalation or stable binding, respectively. Interestingly, this region contains two repetitive elements: an amino acid sequence (residues 23−44) with an almost perfect sequence match (residues 27−39) to the β-ladder DNA-binding wing of the ssDNA-binding protein G5P of bacteriophage fd (*20, 21, 1*), followed by an imperfect but reasonable repeat of this sequence (residues 45−68)(Figure 6). Therefore, two peptides have been synthesized, one of which, R23R (residues 22−44), contains the amino acid sequence homologous to the prokaryotic DNA-binding wing, whereas the other peptide, R25R (residues 44−68), might contain a second DNA-binding wing of lower affinity for nucleic acids within its repetitive sequence. Each of these peptides contains 4 arginine and 2 tyrosine residues and, at first glance, might be expected to bind to oligonucleotides equally well (or equally poorly). In 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, this was indeed the case: both peptides bound to the F−$(dG)_{25}$ oligonucleotide equally well, with an affinity about 20-fold less than the NT1 peptide or intact vimentin. Surprisingly, the binding by R23R, but not by R25R, was resistant to dissociation by the addition of KCl to 150 mM, indicating that either Tyr29 or Tyr37 (or both) contribute to the binding and that both Tyr52 and Tyr60 do not. These observations were corroborated by the results obtained with the deletion proteins. Mutant proteins lacking either region (i.e., NΔ25−38 or NΔ43−68) bound the oligonucleotide quite well at low ionic strength, but only the protein possessing the first DNA-binding wing (i.e., NΔ43−68) bound the oligonucleotide in a salt-stable fashion (Figure 2).

To summarize the conclusions derived from the DNA-binding data (see also Table 3), association of vimentin with nucleic acids depends largely upon both electrostatic interactions and intercalation or stacking interactions. Those residues most important for these interactions include arginine at positions 27, 35, 44, 49, and 63 (with perhaps minor contributions by those at 22, 68, and/or 70) and the tyrosines at positions 29 and/or 37. In addition, hydrogen bonding will probably contribute to the stabilization of the nucleic acid/vimentin complexes, whereby the arginine residues of the head domain may play an important role (*22*). Their tendency to form a pair of hydrogen bonds with the O6 and N7 positions of guanine at least partially explains the selectivity of the arginine-rich head domains of IF proteins for guanine-rich DNAs (*6, 23*).

Table 3: Summary of the DNA-Binding and in Vivo Polymerization Properties of Vimentin, Vimentin Deletion Mutant Proteins, and the Peptides Employed in this Study

protein	DNA binding: electrostatic	DNA binding: intercalation	in vivo polymerization[a]
vimentin	+	+	filaments
T-vimentin	−	−	diffuse distribution, no filaments
NΔ10	+	+	filaments
NΔ13	+	+	filaments and aggregates
NΔ17	+	−	aggregates
NΔ32	+	−	diffuse distribution
NΔ25−38	+	−	filaments
NΔ43−68	+	+	filaments
NΔ25−63	±	−	filaments (short)
NΔ25−68	−	−	diffuse distribution, no filaments
peptides			
NT1	+	+	
Nitrated NT1	+	−	
R23R	+	+	
R25R	+	−	

[a] The data for the polymerization of the NΔ10 through NΔ32 proteins were taken from ref *11* and the data for T-vimentin are unpublished results. None of the peptides are capable of polymerizing into IFs.

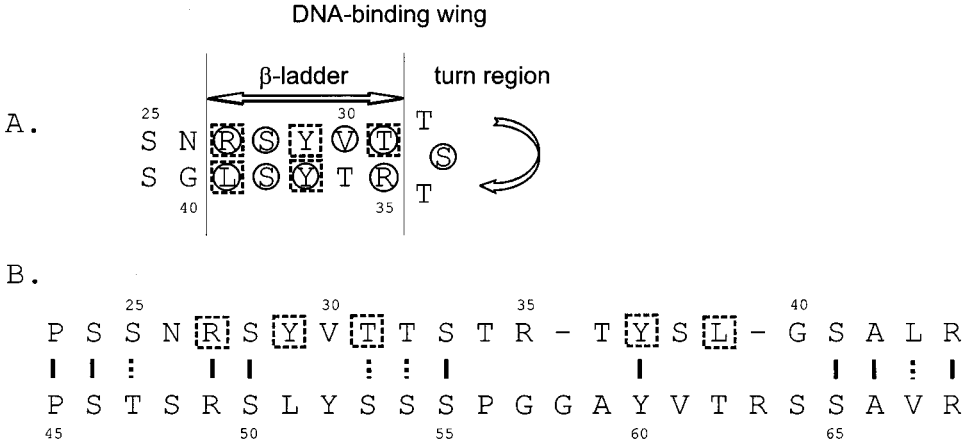

FIGURE 6: Similarity of the sequence of the middle of the N-terminal head domain of vimentin to that of the β-ladder DNA binding wing of the phage fd G5P protein. (A) The vimentin amino acid sequence (residues 25−41, numbers indicated above or below the respective amino acids) is shown folded into the β-ladder DNA binding wing structure. Residues identical or representing conservative substitutions in both proteins are indicated by the open circles. Those residues known to constitute one side of the β-ladder in the fd G5P protein and known or thought to be in contact with DNA are indicated by the squares with a broken outline. (B) Alignment of the amino acid sequence of the regions of the N-terminal head domain of vimentin showing the homology between the first repeat (see A) and the second imperfect repeat of the β-ladder DNA binding wing. These repeats correspond essentially to the peptides R23R (residues 22−44) and R25R (residues 44−68). Identical residues in both repeats are indicated by a solid line and conservative substitutions by a dotted line. Gaps introduced in the turn region and following the β-ladder of the first repeat to maximize the homology at the extreme end of the repeats are indicated by a dash (−).

Many studies have shown that both ends of the N-terminal head domain of vimentin are critically important for IF formation (*24*, *11*, and references therein). We have previously suggested, based on unpublished data obtained with other vimentin deletion mutant proteins that polymerize into morphologically normal IFs, that the middle of the vimentin head domain may loop out from the surface of the IF proper and be available for interaction with other cellular components (including nucleic acids)(*2*). This prediction is supported by experimental data demonstrating that oligo $d(G)_{25}$ binds to vimentin IFs with a basic periodicity of 22−23 nm, which is the same as that observed for the surface structure periodicity of IFs (*25*). The data presented in this paper demonstrate that the regions of the middle of the N-terminal head domain with homology to the DNA-binding wing of the phage fd G5P protein (i.e., residues 27−39 and 49−62) can be individually deleted without any apparent effect on the polymerization properties of the modified proteins. While the structures formed by the NΔ25−63 protein were clearly filamentous in nature, they are shorter and finer than those observed with wild-type vimentin or the NΔ25−38 and the NΔ43−68 proteins, probably because they are not able to laterally align into thicker cables. Since the NΔ25−68 failed to polymerize, it is clear that the deletion of too large a segment of the middle of the N-terminal head domain, rather than the deletion of one or more critical residues, is responsible for this behavior.

Thus, the head domain of vimentin apparently contains 3 subregions: 2 terminal regions (residues 1 to about 17 and from about 70 to the end) responsible, necessary and sufficient for IF polymerization and a central region (roughly residues 20−26 to 69) that probably loops out from the tetramer and IF and may bind to nucleic acids (*14*, *25*) and/or engage in other interactions (i.e., with negatively charged lipid vesicles (*26*−*28*), with membranes (reviewed in *29*), with ribosomes (*30*, *31*), etc.). This looping out may be necessary to bring the evolutionarily conserved N-terminal region of the head domain (*24*) into close proximity and permit its interaction with a specific region of the rod domain of a neighboring protein subunit during IF polymerization (*10*, *32*). It is noteworthy that the N-terminal head domain of GFAP is shorter than that of vimentin by 36 residues (*40*) but is still capable of copolymerization with vimentin (*33*, *34*). GFAP thus might be regarded as a type of natural deletion mutant of type III IF subunit proteins. Yet, looping out of the central region of the N-terminal head domain from the surface of the filament body proper must not be an absolute requirement for the filaments to associate with negatively charged macromolecular structures, such as nucleic acids, lipid vesicles, etc. Previous studies have also shown that, while desmin lacking the first 67 residues (T-desmin, produced by cleavage with thrombin) is itself unable to polymerize into IFs, it can be tolerated in significant quantities when incorporated into desmin IFs (*35*). Employing headless vimentin in cell transfection studies, the fraction of polymerization-incompetent protein subunits tolerated in the cytoskeletal vimentin IF network has been estimated to be about 25% (*36*). This suggests that not every N-terminal head domain of every IF subunit protein is required for IF polymerization and may thus be available for other interactions, e.g., after release from the α-helical rod domains of the filament body by phosphorylation (*37*, *38*) or activation via other posttranslational modifications (e.g., ADP-ribosylation, ref *39*). Of course, in the context of our model (*2*), this would mean that different IFs and different IF subunit proteins, despite being organized into the common 10 nm filament structure, would have different reactivities and different binding partners in vivo. Since the central region of the N-terminal head domain of vimentin may be deleted without altering IF formation and, conversely, the first 13−17 amino acids may be deleted without drastically altering nucleic acid binding (even though filament formation is dramatically altered or abolished [*11*, *24*]), it is clear that these different regions of the head domain possess unique

and independent reactivities and may function, logically, uniquely, and independently.

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