Heterodimeric Associations between Neuronal Intermediate Filament Proteins

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Formation of protein dimers involving α-internexin, peripherin, and the neurofilament (NF) proteins NFH, NFM, and NFL was investigated by partial renaturation of various combinations of individually purified subunits in buffered 2 M urea. Oligomers that were formed were resolved by “blue” native electrophoresis (Schägger, H., Cramer, W. A., and von Jagow, G. (1994) Anal. Biochem. 217, 220–230) modified to include urea in the polyacrylamide gels. Combining this method with Western blot analysis, disulfide cross-linking, and SDS-polyacrylamide gel electrophoresis in the second dimension showed that NFL readily forms significant amounts of heterodimer with NFH, NFM, α-internexin, or peripherin in the presence of 2 M urea. α-Internexin and peripherin also formed heterodimers with NFH or NFM under these conditions. The modified version of blue native gel electrophoresis described here may be useful in monitoring the impact of post-translational modifications and mutations on the dimerization of intermediate filament proteins.

The cytoskeleton of most eukaryotic cells includes an intermediate filament (IF) network assembled from protein subunits expressed in a cell-specific manner (see Ref. 1 for a review). IF proteins belong to a large family of related polypeptides that share a characteristic tripartite domain organization consisting of a conserved —310-amino acid-long α-helical “rod” domain flanked by amino-terminal “head” and carboxyl-terminal “tail” domains of more heterogeneous length and composition (see Ref. 2 for a review). The first step in IF assembly involves dimerization of two identical subunits, as seen with vimentin and desmin (3), or two different subunits, as in the case of keratins (4, 5). This initial dimerization step produces an untagged parallel coiled-coil species (1) that can be chemically cross-linked with the copper phenanthroline (CuP2) reagent in cases where the subunits contain a cysteine residue located in the rod domain (3).

Neuronal cells from cultured embryonic rat dorsal root ganglia (DRGs) are unusual in that they contain at least five different developmentally regulated and co-assembled neuronal IF (nIF) subunits (6). They include α-internexin (7), peripherin (8), and the neurofilament (NF) triplet proteins, which consist of the heavy (NFH), mid-sized (NFM), and low (NFL) molecular mass subunits (9). Among these five nIF proteins, only NFL has been purified and chemically cross-linked as a dimer (3), although disulfide-linked peripherin dimers that occur naturally in vivo have been reported (10). NFs appear to be obligate heteropolymers in vivo (11, 12), and individual NF subunits can also be incorporated into an α-internexin (12), peripherin (13), or vimentin (14, 15) network.

Several lines of evidence support or suggest the existence of NFH/NFL or NFM/NFL heterodimers as the smallest heterogeneous unit of NF assembly. For instance, cross-linking analysis of native or partially disassembled NFs revealed that NFM could be cross-linked to NFL through cysteine residues located in the rod domain, although the approach that was used could not differentiate intra- from inter-coiled-dimer cross-linking (16). Furthermore, Mulligan et al. (17) concluded that the existence of NFH/NFL and NFM/NFL heterodimers could best reconcile their immunoelectron microscopic data with the available literature (see discussion in Ref. 17). Finally, NFH and NFM were found to interact more strongly with NFL than with themselves or with each other in a yeast two-hybrid system paradigm, suggesting that heterodimer formation with NFL is favored over NFH and/or NFM oligomerization (18, 19).

To date, however, the smallest NF hetero-oligomers examined biochemically were reported to be heterotetramers containing equimolar amounts of NFH or NFL with NFL. However, it could not be determined whether these were assembled from individual homo- or heterodimers (20). Moreover, Triton-soluble hetero-oligomers of α-internexin, peripherin, and the NF subunits have been reported, but their precise composition also remains unknown (6). Thus, the precise nature of the smallest hetero-oligomers involving the various nIF proteins is still elusive, due largely to the limitations of experimental approaches capable of resolving and characterizing these small in vivo or in vitro assembly intermediates.

A novel “blue” native polyacrylamide gel electrophoresis (BN-PAGE) system has recently been described in which Coomassie Blue G-250 is used to confer a negative charge to protein complexes, allowing them to be separated on the basis of their molecular mass while preserving their native associations (21). Initial studies conducted in our laboratory showed that BN-PAGE, rendered “semi-native” by inclusion of 2 M urea, resolved nIF proteins into what appeared to be heterocomplexes. Further studies of nIF protein complexes were initiated in which various combinations of individually purified denatured subunits were assembled by dialysis against buffered 2 M urea. Subsequent analysis of assembled protein complexes by 2 M urea-BN-PAGE, together with other techniques, demonstrated that NFL forms heterodimers with NFH or NFM and...
that α-internexin and peripherin can form heterodimers with the individual NF subunits. These results indicate that previously reported interactions between the various nIF proteins (6) may take place at the level of heterodimer formation.

EXPERIMENTAL PROCEDURES

Preparation of Cytoskeletal Extracts—13,000 × g insoluble cytoskeletal extracts of embryonic cultured DRG neurons and PC12 cells were prepared essentially as described (6).

Purification of Neuronal Intermediate Filament Proteins—NFH, NFM, NFL, and α-internexin were isolated as NFs from rat spinal cord as described (22), whereas peripherin was from Triton X-100-insoluble PC12 cell extracts. These preparations were solubilized and boiled for 5 min in SDS sample buffer (2% SDS, 62.5 mM Tris (pH 6.8), 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol) (23) and separated by discontinuous SDS-PAGE in the presence of 15 mM thiglycolic acid to prevent oxidation of the cysteine residues. The proteins of interest were visualized, excised, and electroeluted as described previously (22). The electroeluted material was recovered, and solid urea was added to a concentration of 2 M. These SDS-containing solutions were then applied to individual columns of Extragel detergent-removing matrix (Pierce) equilibrated with a degassed buffer containing 2 M urea, 50 mM Tris (pH 9.0), and 5 mM DTT. Following elution with the same buffer, an aliquot was removed for quantification and verification of protein purity and for SDS assay to ensure that the detergent had been removed (24).

The protein solutions were dialyzed against a buffer containing 6 M guanidine hydrochloride, 50 mM BisTris (pH 7.0), and 5 mM DTT by repeated centrifugations in a Centricon dialysis spin column (Amicon, Montreal, Canada), aliquoted, and stored at –80 °C until further use. Proteins quantified by the bicinchoninic acid method (Sigma) were purified in the absence of reducing agents, and the SDS removal protocol was replaced with dialysis against 50 mM NaCl and 0.01% SDS in the Centricon spin columns. This permitted the removal of Tris, glycine, DTT, and excess SDS, which may interfere with the protein assay.

Cell Culture—Embryonic rat DRGs were dissected, dispersed, and maintained in defined medium as described previously (6, 25).

Partial Assembly of nIF Proteins—The individually purified nIF proteins were mixed (final concentration of 0.1–1.0 mM) and dialyzed in a Microdialyzer apparatus (Pierce) at room temperature for 2.5 h against two to three changes of an extensively degassed buffer containing 2 M urea, 50 mM Tris (pH 7.0) and urea at the concentrations specified in the figure legends.

Disulfide Cross-linking of nIF Protein Complexes—Dialyzed samples of nIF protein complexes were cross-linked through their cysteine residues by adding CuP2 (3, 16, 26) from a freshly prepared concentrated stock solution to a final concentration of 15 mM. Cross-linking was carried out for 20 min at room temperature and stopped by adding EDTA to 5 mM and iodoacetamide to 50 mM (modified from Refs. 3, 16, and 26).

Analysis of nIF Protein Complexes by BN-PAGE—nIF protein complexes were resolved by BN-PAGE as described (21) with the following modifications. Stacking gels were omitted, and amineacryl acid in the gels was replaced with 2 M urea. 5% glycerol was added to gel solutions used to pour gradient gels. The upper tank buffer contained 0.01% Coomassie Blue G-250; the 9-fold concentrated loading buffer contained 50 mM BisTris (pH 7.0), 2 M urea, 2.5% Coomassie Blue G-250, and 50% glycerol. Gels were cast in a Bio-Rad minigel apparatus and electrophoresed at 100 V. Disassembly of assembled protein complexes was achieved by heating the samples to 56 °C for 4 min immediately prior to electrophoresis, which effectively prevented reassembly. Dissociation of disulfide-linked protein complexes was achieved by including 10 mM DTT in the heated samples. For second dimension gel electrophoresis, lanes were cut out of the gels after BN-PAGE and incubated in 1.5-fold concentrated SDS sample buffer with or without 5% β-mercaptoethanol as described in the figure legends.

SDS-Polyacrylamide Gel Electrophoresis—Discontinuous gel electrophoresis of the samples on 4.5 or 6% SDS-polyacrylamide gels was performed as described (23). Electrophoresis on 4% Weber and Osborn-type gels (27) was performed essentially as described (28), except that regular Tris-containing SDS sample buffer (see above) was used for dilution of samples. Western Blot Analysis—After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Montreal) as described (6). After electroblotting of proteins resolved by BN-PAGE, PVDF membranes were rinsed in methanol to remove Coomassie Blue. Proteins were detected with specific monoclonal antibodies (mAbs) against NFL (NB4), NFM (NN18), and NFH (NS22) (Sigma)

RESULTS

nIF Proteins from Cultured DRG Neurons Resolved by 2 M Urea-BN-PAGE Show Evidence of Heteromeric Associations—We previously reported that α-internexin, peripherin, and the NF triplet proteins in cultured DRG neurons were associated (6), but the methods used could not accurately determine the precise oligomeric arrangement of these proteins. Further studies in our laboratory showed that nIF extracts resolved by BN-PAGE in the presence of increasing amounts of urea contained heterocomplexes of nIF proteins. In Fig. 1, a DRG cytoskeletal pellet extracted with 2 M urea was electrophoresed using a semi-native version of BN-PAGE, which included 2 M urea in the gel. Development of the resulting Western blots with mAbs against the individual nIF proteins showed seven major bands, some of which were immunoreactive with two of the mAbs. Based on the staining intensity and the relative mobilities of the various species, protein complexes 1 and 2 appeared to be hetero-oligomers of NFL with NFM and NFH, respectively, whereas complex 4 in the anti-NFH lane appeared to be an NFM dimer. Complex 3 in the anti-NFH lane was likely the hypophosphorylated NFH-containing equivalent of complex 1 since it was partially prominent in extracts from the cell body-enriched fraction obtained from localized DRG cultures (data not shown), which we have shown to contain mostly this form of NFH (6). The broad region designated as 6 contained species staining with mAbs against NFM, α-internexin, and peripherin, suggesting the presence of homo- and/or
heterodimers. Complex 4 in the anti-NFM lane, complex 5 in the anti-NFL lane, and complex 7 in the anti-peripherin lane could not be identified unambiguously. Initial cross-linking studies with further resolution in a second SDS-PAGE dimension appeared to confirm some of the associations described above, and further studies were undertaken to fully characterize these and other small nIF protein complexes. This was achieved by mixing various combinations of individually purified nIF subunits in the presence of 6 M guanidine hydrochloride, dialyzing against buffered 2 M urea, and resolving the resulting complexes on semi-native BN-PAGE gels prepared with the same buffer as the dialysis solutions.

**Purified NFL Forms Heterodimers with NFH and NFM** — As shown in Fig. 2 (lanes 1 and 3), purified NFH and NFM migrated on 2 M urea-BN-PAGE as single bands with apparent molecular masses of 260 and 214 kDa, respectively. Heating to 56 °C prior to electrophoresis did not affect their mobility (lanes 2 and 4), indicating that NFH and NFM migrated as monomers in this gel system with anomalously high apparent molecular masses, as is also observed on SDS-PAGE (32). Gradual increases in the gel urea concentration to 8 M failed to produce smaller species, confirming the identity of the NFH and NFM bands as monomers (data not shown).

NFH dialyzed and resolved on 2 M urea-BN-PAGE (Fig. 2, lane 5) yielded two bands with apparent molecular masses of 226 and 94 kDa. Heating the dialyzed NFL to 56 °C prior to electrophoresis (lane 6) converted almost all of the larger species to the more rapidly migrating form. Disulfide cross-linking of the NFL homo-oligomer through the unique cysteine residue in the α-helical domain (14) using CuP2 (3) did not alter the migration pattern (lane 7) and effectively protected a significant portion of the NFL 226-kDa species from heat-induced conversion to the 94-kDa species (lane 8), indicating that these were homodimers and monomers, respectively. The CuP2-induced cross-links could be broken by including 10 mM DTT in the heated samples (lane 9), as would be expected for disulfide bonds.

When mixtures of NFL with NFH or NFM were analyzed by 2 M urea-BN-PAGE, the bands previously identified in the lanes containing these proteins alone were all present. In addition, new complexes appeared with apparent molecular masses of 360 kDa for the NFH/NFL mixture (Fig. 2, lane 10) and 328 kDa for the NFM/NFL mixture (lane 15), indicating the formation of NFH/NFL and NFM/NFL hetero-oligomers under these conditions. As was seen with the NFL homodimer, these hetero-oligomers were heat-labile (lanes 11 and 16); they could be stabilized by CuP2 cross-linking (lanes 13 and 18); and the disulfide cross-links were reduced with DTT (lanes 14 and 19).

Given the apparent anomalous migration of nIF proteins in this gel system and because both NFH and NFM each contain more than one cysteine residue (30, 31), two complementary approaches were used to confirm that the hetero-oligomers formed between NFL and NFH or NFM were heterodimers. In Fig. 3, triplicate samples of NFH/NFL (panel A) and NFM/NFL (panel B) hetero-oligomers were prepared and resolved essentially as described for Fig. 2. The large amount of material used allowed direct visualization of all protein species in the first (2 M urea-BN-PAGE) dimension as they appeared as Coomassie Blue G-250-stained bands against a lighter blue background. The protein bands were excised, briefly equilibrated with SDS sample buffer, resolved in the second dimension by discontinuous SDS-PAGE alongside standardized mixtures of NF subunits, visualized by Coomassie Blue R-250 staining, and quantified with the BioImager apparatus. As shown in Fig. 3, the NFH/NFL and NFM/NFL hetero-oligomers purified by BN-PAGE in the first dimension broke down in the second SDS-containing dimension to give essentially equimolar amounts of NFH and NFL (ratio = 1.16 ± 0.02) or NFM and NFL (ratio = 1.06 ± 0.02) (mean ± S.D.). These results clearly demonstrate that the observed hetero-oligomers are equimolar assemblies of their NF subunit constituents.

In Fig. 4, NFH/NFL (panel A) and NFM/NFL (panel B) hetero-oligomers were formed, cross-linked, and resolved by 2 M urea-BN-PAGE essentially as described for Fig. 2. Some lanes were blotted onto a PVDF membrane and reacted with the anti-NF mAbs to reveal the positions of the various protein complexes in the first dimension as indicated at the top of Fig 4. Other lanes were cut out of the gels, equilibrated with SDS sample buffer without reducing agent, and electrophoresed in the second dimension on a Weber and Osborn-type gel (27), which provided a better estimate of the molecular mass of cross-linked proteins than was obtained with Laemmli-type gels (data not shown), as previously reported (3, 16, 28, 33, 34).

Following transfer to a PVDF membrane, the blots were probed with an anti-NFL mAb (Fig. 4, lower portions of panels A and B), erased, and reprobed with an anti-NFH mAb (upper portion of panel A) or an anti-NFM mAb (upper portion of panel B). The cross-linked NFH/NFL hetero-oligomer seen in the first (2 M urea-BN-PAGE) dimension (H/L) resolved into three bands in the second SDS-PAGE dimension. The spot labeled H/L in the second dimension reacted with both anti-NFH and anti-NFL mAbs (upper and lower portions of panel A, respectively), confirming its heteromeric character. Non-cross-linked NFH (H)
and NFL (L) were also present in this second dimension, as could be expected from examining the heated cross-linked samples in Fig. 2 (lanes 13 and 18). Homodimeric NFL from the cross-linked sample seen in the first dimension (L/L) gave rise to some cross-linked (L/L) and monomeric (L) NFL in the second dimension, whereas the NFH and NFM species that were monomeric in the first dimension (H and L) remained so in the second dimension (H and L). Similar results were obtained for the NFH/NFL mixture (panel B), although cross-linked dimeric NFL (L/L) could not be adequately resolved from monomeric NFL (M), apparently due to upward streaking of the latter. It was also observed that the relative amount of cross-linked hetero-oligomer was lower on the Weber and Osbourn-type gels than would be predicted from the data in Fig. 2 (e.g. lanes 13 and 18). This was a reproducible yet unexplained limitation of this gel system that was not observed when second dimension electrophoresis was carried out in a discontinuous SDS-PAGE system (data not shown). The apparent molecular masses of the various species detected were determined and are listed in Table I. The values for cross-linked NFH/NFL, NFM/NFL, and NFL/NFL were within 3% of the summed molecular masses of their monomeric constituents, thereby confirming their dimeric character. Taken together, the results in Figs. 2–4 and in Table I conclusively demonstrate that, under the conditions described here, NFH/NFL and NFM/NFL heterodimers and NFL/NFL homodimers are formed and can be resolved by 2 M urea-BN-PAGE.

α-Internexin and Peripherin Form Heterodimers with NF Subunits—In Fig. 5, α-internexin and peripherin were tested for their ability to dimerize alone or in combination with other nIF proteins. The experiments were carried out essentially as described for Fig. 2, except that cross-linking and heating of some species was omitted. Some of the previously characterized NF species are shown again to provide useful molecular mass landmarks. Based on the relative mobilities of the protein complexes, both α-internexin and peripherin formed homodimers (~159 and ~143 kDa in lanes 5 and 7, respectively) that broke down to the monomeric species upon heat treatment (~73 and ~66 kDa in lanes 6 and 8, respectively). The staining intensity of the heat-treated peripherin sample was reproducibly lower than that of the unheated sample (lanes 8 and 7, respectively), apparently due to in-gel dimerization of the monomeric species, leading to extensive streaking and comcomitant loss of signal. The significant amount of dimeric peripherin in the heated sample and the virtual absence of monomeric peripherin in all of the other peripherin-containing lanes (lanes 7, 9, and 14–16) confirm its very strong tendency to dimerize under the conditions used. The various dimeric species obtained when mixing α-internexin and peripherin (lane 9) could not be resolved adequately on these gels, and cross-linking experiments failed to show any heterodimeric interactions between these two proteins (data not shown). Based on the relative mobility of the protein complexes, heterodimers containing α-internexin or peripherin with NFH (~356 kDa) or NFM (~320 kDa) (lanes 10, 11, 14, and 15, respectively) were also observed. Although the relative yields of these heterodimers were much lower than those observed for the NFH/NFL and NFM/NFL species, markedly greater amounts could be generated by dialyzing and resolving the samples in 0.05 M urea instead of 2 M urea (data not shown). It is not known why the α-internexin/NFH and α-internexin/NFM heterodimers in Fig. 5 migrated as doublets, although protein degradation seems unlikely since it was not observed in the purified subunit preparations (data not shown). Mixing α-internexin or peripherin
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...strength buffers, sometimes containing urea or guanidine in a variety of means, all revolving around the use of low ionic strength buffers. Sometimes, reassembly of NFs (37, 38), centromeres coupled with removal of urea or guanidine, if reassembly of IF proteins from concentrated guanidine solutions (3–5, 20, 35, 36). IF protein oligomers generated from buffered 2M urea and have a practical and widely accessible technique for resolving small oligomeric assembly intermediates. We have now shown that the recently reported BN-PAGE procedure (21), modified to include urea, may provide direct and unambiguous separation of IF protein complexes and allows multiple combinations of IF proteins to be resolved in parallel. To this end, Fig. 2 provides direct evidence for heterodimer formation betweenNFL and NFH or NFM, whereas Fig. 5 further shows heterodimeric associations between α-internexin or peripherin and the individual NF subunits.

Small IF assembly intermediates have been generated by a variety of means, all revolving around the use of low ionic strength buffers, sometimes containing urea or guanidine in the 2–3 M range. Such buffers have been used either as a diluent of whole IFs (16) or as the dialysis component for limited reassembly of IF proteins from concentrated guanidine or urea solutions (3–5, 20, 35, 36). IF protein oligomers generated under these conditions have been considered to be valid assembly intermediates since addition of physiological salt concentrations coupled with removal of urea or guanidine, if required, leads to the formation of normal-appearing IFs. Moreover, reassembly of NFs (37, 38), α-internexin (7, 39), and peripherin (40) from urea-containing buffers has been well documented. More specifically, NFL and NFM have been successfully co-assembled into native-like filaments by stepwise dialysis from guanidine solution to a physiological buffer, including an intermediate step involving a buffered 2 M urea solution similar to ours (20). Taken together, these data indicate that other nIF protein oligomers formed under such conditions would be valid assembly intermediates.

A previous report described the resolution of NF subunit heterotetramers containing equimolar amounts of NFL with NFM or NFM on “native” gels containing 2 M urea (20). In contrast, our study using the BN-PAGE system revealed that dimers were the predominant species under similar conditions. It is possible that the presence of Coomassie Blue G-250 destabilizes tetrameric species, thus favoring the dimers that we observed, although such effects were not reported by Schägger et al. (21). Alternatively, the bovine material used by Cohlbeg et al. (20) may form complexes that are more stable in 2 M urea than those formed by the rat nIF proteins utilized in our work. However, neither of these possibilities affects the present conclusions relating to heterodimer formation.

NFH and NFM did not interact with themselves (Figs. 2 and 5) or with each other (data not shown), consistent with the many reports indicating that NFM and NFH do not assemble or co-assemble significantly in vitro (37, 38, 41) or in vivo (11, 12) and further indicating that the interactions we did observe were not likely due to nonspecific interactions between related α-helical rod domains.

The first step in IF assembly involves the formation of an unstaggered parallel coiled-coil dimer (2, 42). Residues in positions a and d in the heptad repeats making up the α-helices are believed to form inward-facing hydrophobic seams, whereas the other residues face outward (reviewed in Ref. 43). NFL contains a unique cysteine residue located in a d position within a heptad repeat in the rod domain (14). Disulfide cross-linking of NF homodimers and other IF protein dimers sharing this conserved residue has been well documented and has provided an important argument favoring the unstaggered parallel coiled-coil model of IF homodimer structure (3, 43). However, disulfide bond formation between NFL and NFH or NFM under the conditions used here is somewhat puzzling. Indeed, the location of the closest cysteine residue in the rod domain of NFH in the linker region L2, 70 residues removed from the cysteine residue inNFL, appears to preclude an unstaggered alignment of these proteins. A similar problem involves the cysteine in NFM occupying the a position in a heptad, 41 residues away from the cysteine inNFL. Yet, Fig. 3 clearly demonstrated that cross-linked NFL/NFH and NFL/NFM dimers can be resolved as such in the 2 M urea-BN-PAGE dimension, raising the possibility that, in contrast to other IF proteins characterized so far (1–3, 42), NF subunits associated in a coiled-coil heterodimer may be in a staggered arrangement. This interpretation appears unlikely considering that some twisting of the helices would also be required to adequately align the cysteine residues involved in the cross-linking of NFM or NFH to NFL. A more likely explanation may be that...
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the 2 M urea-containing buffer used here allows for slippage and flexibility within the coiled-coil structure, which would permit interactions between cysteine residues that are widely separated in the normal unstaggered alignment. According to this view, the mobility of subunits in such loosely assembled coiled-coil dimers would still allow for more efficient intradimer cross-linking compared with cross-linking between unassembled random collision complexes, which were never observed in these experiments.

α-Internexin and peripherin were shown in Fig. 5 to form heterodimers with NFH or NFM, suggesting that co-assembly of these proteins can take place at the dimer level. The comparatively greater propensity for NFL to form heterodimers with NFH and NFM further suggests that it is the preferred partner for these proteins. However, heterodimeric association of α-internexin or peripherin with the NF subunits may be important for cytoskeletal reorganization during development and axonal regeneration. α-Internexin is the predominant nIF protein in developing central neurons (39, 44), whereas peripherin expression is up-regulated in regenerating large-caliber protein in developing central neurons (39, 44), whereas peripherin with the growing axon (46). The ability of NF proteins to dimerize with α-internexin and peripherin could play an important role in allowing a gradual transition to take place from a more plastic α-internexin- or peripherin-containing network to a stable NF-enriched IP network in the mature axon (47).

The modified version of BN-PAGE described here is a widely accessible, economical, and versatile method that allows several different samples to be analyzed in parallel. Future work in our laboratory will focus on monitoring early nIF protein assembly intermediates and on assessing the effects of mutations or post-translational modifications known or suspected to affect IP assembly.

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