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Truncated desmin in PtK2 cells induces desmin–vimentin–cytokeratin coprecipitation, involution of intermediate filament networks, and nuclear fragmentation: A model for many degenerative diseases

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ABSTRACT **The earliest expression of truncated desmin in transfected PtK2 cells results in the formation of dispersed microprecipitates containing not only the truncated desmin, but also endogenous vimentin and cytokeratin proteins. Desmin microprecipitates without vimentin or vimentin microprecipitates without desmin are not observed. The microprecipitates involving cytokeratin invariably are also positive for desmin and vimentin. Over time, the precipitates enlarge into 1- to 2-μm spheroids and then fuse into amorphous chimeric juxtanuclear masses that can occupy >30% of the cell volume. Concurrently, first the vimentin and then the cytokeratin networks are resorbed. The chimeric precipitates are not recognized or marked for degradation by the lysosomal system. Ultimately the cell nucleus fragments and the cell dies. Similar protein complexes appear in many human and animal pathologies, suggesting that a similar protein-precipitation sequence initiated by the introduction of a mutationally or environmentally altered protein molecule is at work.**

Since the identification of intermediate filaments (IFs) in developing myotubes >25 yr ago (1), >30 IF proteins have been identified. The genes for most of these have been cloned, regulatory sequences have been delineated, and the conditions favoring the polymerization of these proteins into elongated 10-nm filaments have been studied (2–7). Still, with rare exceptions (e.g., refs. 8 and 9), little is known of the intracellular interactions and functions governed by these filaments. For example, it is still unclear why newborn, postmitotic myoblasts up-regulate desmin synthesis before that of any of the myofibrillar proteins (10–14) or why desmin IFs shift from a longitudinal orientation in early myotubes to a transverse orientation with a sarcomeric periodicity after myofibrillogenesis is advanced (10, 15).

To study the process of protein incorporation into IFs, Schultheiss *et al.* (16) transfected replicating myogenic cells with a truncated desmin cDNA. They reported that the truncated desmin was not incorporated into the cell's preexisting chimeric desmin$^+$/vimentin$^+$ network. Rather, dispersed microprecipitates formed and grew into desmin$^+$/vimentin$^+$ spheroids ≈2 μm in diameter. The growth of the spheroids was correlated with the dissolution of the preexisting desmin$^+$/vimentin$^+$ IF network.

To study this process further, we have similarly transfected PtK2 cells, which typically assemble separate vimentin and cytokeratin networks but do not synthesize desmin. We describe here a sequence of events in which the altered desmin progressively diverts the endogenous IF proteins into massive precipitates that lead to cell death.

A number of genetic and environmentally induced degenerative diseases of skin, liver, and nerve exhibit intracellular structural changes that are remarkably similar to the pathology induced by altered desmin in PtK2 cells. We suggest that these pathologies may be driven by an analogous mechanism in which the introduction of an altered protein acts to aggregate and precipitate related, normally interacting proteins.

MATERIALS AND METHODS

Full-length desmin cDNA was truncated at the unique *Cvn* I restriction endonuclease site, within segment 2 of the highly conserved α-helical rod domain, and subcloned as described (16). The truncated protein contains 272-amino acid residues compared with 468 residues for full-length desmin.

PtK2 cells (American Type Culture Collection) were cultured onto collagen-coated Aclar coverslips (Pro-Plastics, Linden, NJ) in 35-mm tissue culture dishes. Cells were plated at an initial density of 10^5 cells per dish in Eagle's minimal essential medium/10% fetal calf serum. One day after plating, the cells were transfected with 2 μg of DNA by standard calcium phosphate precipitation methods (16, 17).

Monoclonal antivimentin antibody (clone Vim 3B4) was purchased from Boehringer Mannheim. The polyclonal antidesmin used does not react with vimentin (13). Polyclonal anticytokeratin, from T. Sun of New York University, does not react with other IF proteins. Polyclonal antibodies to nonmuscle myosin heavy chain (MHC:BioTechnology, Stroughton, MA) and monoclonal antibody anti-α-actinin (clone BM 75.2, Sigma) stain stress fibers. The monoclonal antibody antiubiquitin was obtained from Chemicon. Polyclonal antitubulin was purchased from ICN. Rhodamine–phalloidin was purchased from Molecular Probes. Affinity-purified fluorescein isothiocyanate- and rhodamine-conjugated goat anti-rabbit IgG and anti-mouse IgG and IgM were purchased from Jackson ImmunoResearch.

The problem of bleed-through in the fluorescence microscope was minimized by running duplicate series. In one series, each of the two primary antibodies was stained with either a rhodamine- or a fluorescein-conjugated secondary antibody. In the second series, the same primary antibodies were stained in the opposite fashion. Thus each antigen was localized by the use of fluorescein- and rhodamine-conjugated secondary antibodies. Confocal images were collected, as described in ref. 16, by using Texas Red secondary antibodies. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Sections for EM were prepared as described (1).

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Abbreviations: IF, intermediate filament; DAPI, 4′,6-diamidino-2-phenylindole.

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RESULTS

Well-spread PtK2 cells assemble robust, widespread vimentin and cytokeratin networks (Fig. 1 *a* and *b*). Neither network stains with antidesmin. After transfection with truncated desmin cDNA, between 5 and 40% of the cells in each dish elaborate a modified desmin protein that binds antibodies to desmin. The expressed desmin produces a characteristic sequence of events, as follows.

Within 12 hr, the transfected cells display large numbers of variably sized, dot-like structures (Fig. 1 *c* and *d*). All the dots are chimeric, binding both antidesmin and antivimentin. Neither desmin$^-$/vimentin$^+$ nor desmin$^+$/vimentin$^-$ dots have been observed. The dots emerge at random throughout the cytoplasm; their appearance near the nucleus does not precede their appearance at the cell periphery. At this early time after transfection, ≈25% of the transfected cells suffer a modest reduction in extent of the vimentin network, whereas the cytokeratin network is unimpaired (see below).

At 24 hr after transfection, the inclusions have increased in number and size. A gradient of chimeric inclusions appears; 1- to 2-μm spheroidal bodies immediately around the nucleus trail off to smaller dots at the cell periphery. The PtK2 spheroids physically resemble the desmin$^+$/vimentin$^+$ bodies seen in myotubes infected with the same cDNA (16) but differ in that 60–70% also contain cytokeratin (Fig. 1 *e* and *f*). Concurrent with the growth of spheroids, a dramatic overall thinning and retraction of the vimentin network toward the cell center occurs. The cytokeratin network is less drastically reduced (compare Fig. 1 *e* with *f*). Changes in both IF networks are selective and not due to some generalized cytotoxicity; double-staining with antibodies to actin, nonmuscle myosin heavy chains, α-actinin, vinculin, and tubulin document normal morphological integrity of the stress fibers and the microtubules (data not shown).

Within 48 hr after transfection, many of the spheroids fuse into massive, irregularly shaped complexes (Fig. 2 *a* and *c*). Confocal reconstructions of these bodies give the impression of links in a coiled chain (data not shown). These are often juxtanuclear in location but not always. The massive links can occupy >30% of the cell volume and can measure up to 15 μm in length and 6 μm in thickness. The convoluted links costain precisely with antibodies to desmin and vimentin. Anticytokeratin generally stains the more superficial regions (Fig. 2*c*). Whether this reflects inaccessible cytokeratin epitopes or involves other factors remains to be seen.

Additional major alterations appear in cells with prominent chimeric, juxtanuclear links. Over 95% (n = 200) either lose their vimentin network totally or retain only small numbers of short, scattered filaments (Figs. 1*e* and 2*a*), while the cytokeratin network exhibits significant, although less extensive, reduction (Figs. 1*f* and 2*c*). In the 48- to 96-hr

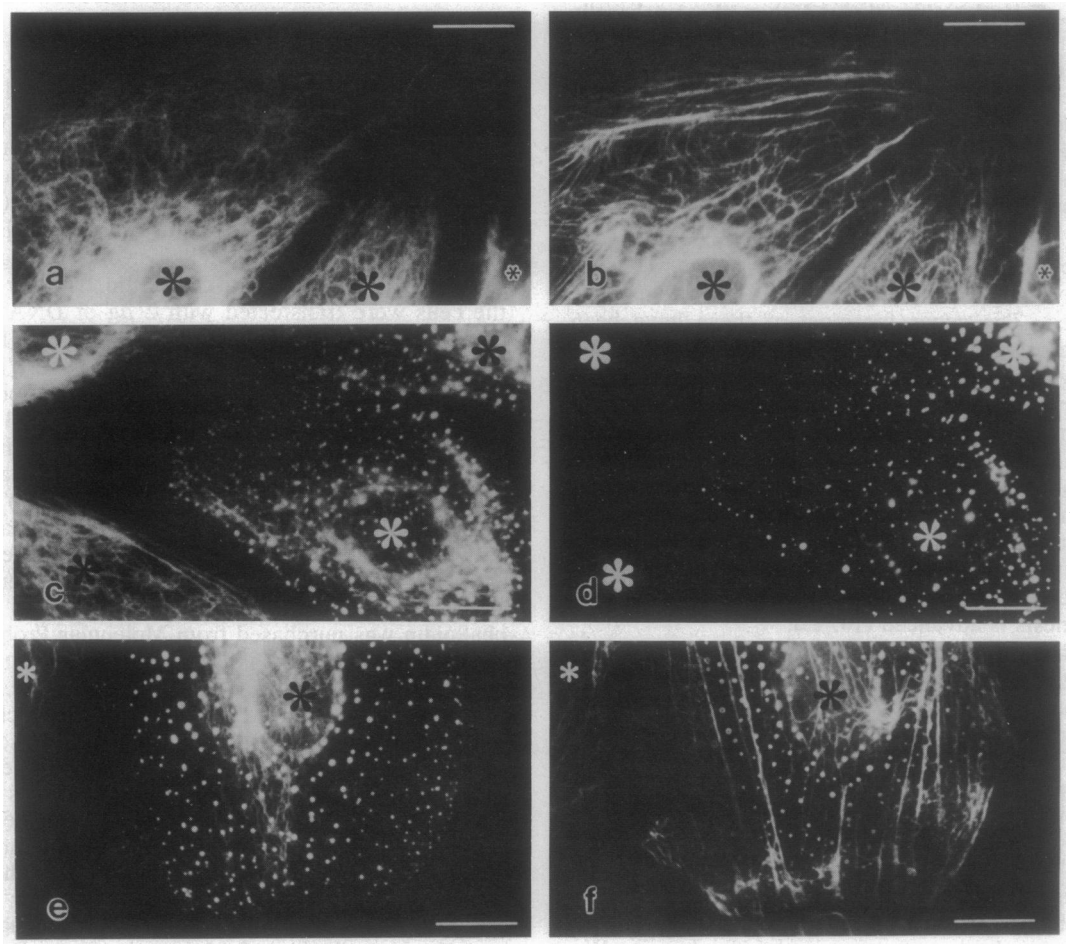

FIG. 1. (*a* and *b*) Fluorescence micrographs of three PtK2 cells double-stained with antibodies to vimentin (*a*; rhodamine channel) and cytokeratin (*b*; fluorescein channel). Both IF networks extend well into the cell periphery. Asterisks mark same cells. (*c* and *d*) Micrographs of four double-stained PtK2 cells 12 hr after transfection. Cells in *c* were stained with antivimentin (fluorescein channel), and cells in *d* were stained with antidesmin (rhodamine channel). Note in the two transfected cells (asterisks at right) the size-gradient and widespread distribution of the desmin$^+$/vimentin$^+$ dots and small spheroids. Contrast the virtual absence of the vimentin IF network in these cells with the vimentin IF network in the two nontransfected cells at left. Cells in *e* and *f* were double-stained with antivimentin (*e*; rhodamine channel) and anticytokeratin (*f*; fluorescein channel). The resorption of the vimentin IF network at this stage, 48 hr after transfection, is more advanced than that of the cytokeratin network. Asterisks mark adjacent nontransfected cells. (Bars = 10 μm.)

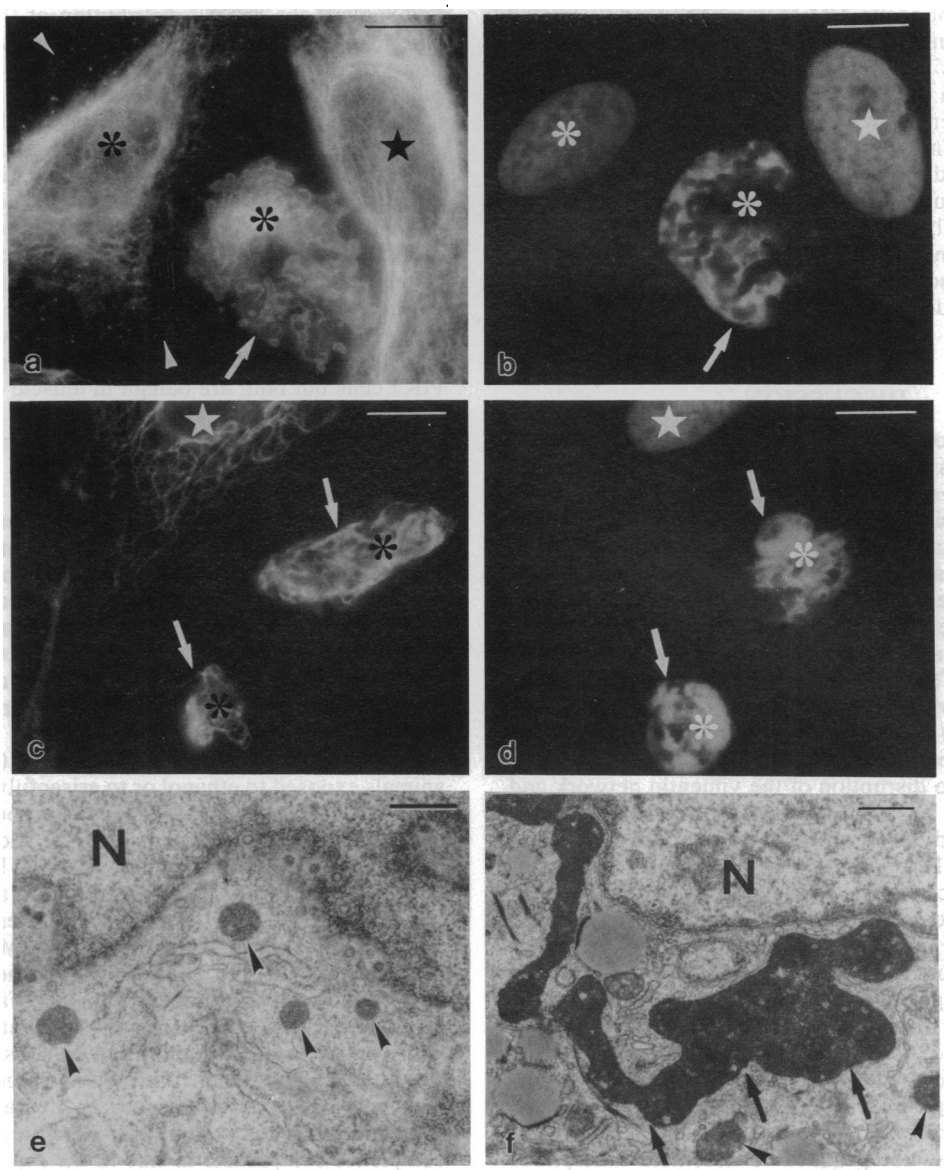

FIG. 2. (*a–d*) Illustration of the high correlation of presence of juxtanuclear links with total loss of vimentin IFs, partial loss of cytokeratin IFs, and pycnotic nuclei. (*a*) Distribution of vimentin (fluorescein channel) in three cells. (*b*) Nuclei of these cells (DAPI). The arrowheads indicate the barely detectable chimeric dots characteristic of cells just beginning to express the truncated desmin. The vimentin network is still intact in this cell, as is its nucleus. The arrow points to a massive juxtanuclear link in a cell in a later transfection stage. Note that the vimentin IF network has totally disappeared, and its nucleus is pycnotic. The star marks an untransfected cell; its IF network and nucleus are normal. (*c*) Cytokeratin distribution in three cells. (*d*) The respective nuclei of the three cells of *c*. Arrows mark two cells in a late stage of transfection. Both cells have totally resorbed their vimentin IF network (data not shown). The star indicates an untransfected cell with a typical cytokeratin pattern and nucleus. (Bars = 10 μm.) (*e* and *f*) EM sections, illustrating differences in size, fine structure, and overall geometry between chimeric dots and juxtanuclear links. Portions of the nucleus (N) are at the top in both *e* and *f*. Dots (arrowheads) and spheroids (not shown) are invariably globular. The amorphous opaque juxtanuclear links in *f* (arrows) are irregularly shaped. Neither dots, spheroids, nor links are bounded by limiting membranes. (Bars = 0.5 μm.)

posttransfection series >80% of the cells with chimeric links display a variety of nuclear and cytoskeletal abnormalities. Lobulated and honey-combed DAPI-positive nuclear masses are often bisected by large, irregularly shaped chimeric links (Fig. 2 *b* and *d*). These micrographs give the impression that the convoluted links deform the nuclei physically. Such cells are no longer spread, have lost their cell–cell junctions and adhesion plaques, and have retracted from the substrate (Fig. 2 *a* and *c*). Invariably when cells with distorted nuclei are stained with antibodies to actin, α-actinin, myosin heavy chains, vinculin, or tubulin, they prove to lack stress fibers, adhesion plaques, and even microtubules. In any given set of experiments ($n = 4$) the cells surviving 96 hr after transfection fall to 20–35% of those surviving in the corresponding 48-hr series. Although formally possible, it is difficult to believe that cells with pycnotic nuclei recover.

Fig. 2*e* shows an electron micrograph of a section through chimeric dots, and Fig. 2*f* shows a section through a small region of a juxtanuclear amorphous link. The ultrastructure of the dots and spheroids is indistinguishable from those assembled in myotubes (16), but chimeric links have not been observed in myotubes. No structures at all similar to those illustrated in Fig. 2 *e* and *f* have been observed in untransfected PtK2 cells or in cells transfected with full-length desmin (unpublished data).

It might be anticipated that partially denatured or misfolded desmin molecules would be subjected to rapid proteolysis by the cytosolic degradative systems of the cell. This

proteolysis does not occur. EM sections show no lysosomal membranes surrounding chimeric dots, spheroids, or juxtanuclear complexes (Fig. 2 *e* and *f*). Further, there is no obvious increase in the number of autophagosomes in transfected cells or even in those with fragmented nuclei. The possible involvement of the ubiquitin-dependent degradative pathway was tested for by staining cells with antiubiquitin. The antibody did not stain any of the chimeric structures preferentially. Lastly, it is worth stressing that the expression in PtK2 cells of truncated myosin heavy chains, light chains, or α-actinin does not result in chimeric dots, spheroids, or links; nor do they disrupt either the vimentin or cytokeratin IF networks (17).

DISCUSSION

A variety of physiological signals disrupt vimentin and cytokeratin networks into aggregates that generally can be cleared rapidly and recycled to form an intact IF network (1, 18–22). A nonphysiological dissociation of IF fibers due to the introduction of altered IF proteins has been noted before by Fuchs (23–25) and by Cleveland (26, 27) and their coworkers. The former group demonstrated that the expression of truncated cytokeratin disrupts the cytokeratin network in cultured skin cells and in the basal cells of the epidermis of transgenic mice. They also noted that a similar condition occurs in patients with epidermolysis bullosa simplex. Cleveland's group found that the expression in fibroblasts of the middle neurofilament isoform (NF-M) with deletions in the rod domain leads to disruption of the vimentin network and the production of chimeric vimentin$^+$/NF-M$^+$ inclusions similar in size and distribution to those described here. Schultheiss *et al.* (16) reported that in myogenic cells truncated desmin induced myriad spheroids and concurrent resorption of the preexisting desmin$^+$/vimentin$^+$ IF network. The spheroids were not bounded by membranes and escaped proteolytic breakdown.

Although still fragmentary, available data suggest that the response of a cell to exogenous cytoskeletal molecules is markedly influenced by the on-going differentiation program and cytoplasmic milieu of that cell, as well as by the conformation of the exogenous protein. For example, although induced by *truncated* desmin to assemble numerous spheroids, myotubes do not proceed to assemble amorphous juxtanuclear links, their nuclei are not fragmented, and they can be maintained for >3 weeks in culture. Furthermore, myotubes transfected with a *full-length* desmin-encoding vector fail to display any detectable changes in their desmin$^+$/vimentin$^+$ IF network (16). In contrast, when the same full-length desmin is expressed in PtK2 cells, the exogenous protein is rapidly (<12 hr) incorporated throughout the entire extended vimentin IF network (16, 28, 29). Nascent full-length desmin does not form dots, spheroids, or links. Nevertheless, over the next 10–20 hr the IF network consisting of vimentin and full-length desmin retracts, not into a juxtanuclear link, but into a juxtanuclear bolus made up of thousands of intact, intertwining long IFs that exclude all organelles, including microtubules, Golgi bodies, microfilaments, ribosomes, lysosomes, and mitochondria. These aggregates of packed aligned IFs are indistinguishable from the meandering IF-cables induced by Colcemid in a variety of cell types (1, 16, 18, 30, 31). In brief, although both the truncated and full-length desmin disrupt (*i*) the vimentin and then (*ii*) the cytokeratin IF networks in PtK2 cells, the structures they initially associate with and the structures they eventually form differ markedly.

This report focuses on the extraordinary temporal and spatial events initiated by an assembly-incompetent desmin expressed in PtK2 cells. The submicroscopic coprecipitates of truncated desmin and endogenous vimentin promptly trap and subvert the overall distribution of the native IF molecules. The coprecipitates, which probably involve dimerization between full-length vimentin and truncated desmin, out-compete the IF networks for soluble tetramers. This action, followed by normal turnover, leads to the involution of both IF networks. The entrainment of the cytokeratins by these chimeric complexes exhibits an option for interactions among types I, II, and III IFs not revealed by other types of *in vivo* or *in vitro* experiments (3–7). These interactions are probably, in part, a secondary consequence of the disruption of the endogenous vimentin network. Ultimately the uncontrolled growth of these chimeric complexes results in nuclear fragmentation and cell death. It is important to learn more of why both full-length and truncated desmin are virulent in PtK2 cells but both are benign in muscle cells, as well as to learn more of the causal events that lead from microprecipitates scattered throughout the cytoplasm to nuclear lesions and death.

The same kind of protein-precipitation syndrome appears at work in a variety of degenerative diseases that have not before been proposed to be due to a common cause. (*i*) As previously noted, the basal cells of patients with epidermolysis bullosa simplex exhibit greatly reduced cytokeratin IF networks and massive cytokeratin aggregates that are similar in structure to those reported here. Inspection of the relevant electron micrographs of such epidermal cells indicates that the aggregates are not bounded by limiting membranes and the cell population does not appear to contain increased numbers of autophagosomes (25, 32–35). (*ii*) Hepatocytes of animals exposed to ethanol or to griseofulvin, a microtubule-depolymerizing agent, assemble numerous Mallory bodies (36, 37) that are reminiscent of those shown here, by both light and electron micrography. Mallory bodies contain antigens in addition to cytokeratins, they are not membrane bound, and the cells that assemble them are not rich in multivesicular bodies. Hepatocytes with Mallory bodies have been termed "empty cells" because the rich cytokeratin network normally present has been lost. (*iii*) Lewy bodies are associated with neuron loss in the substantia nigra of patients with Parkinson disease and other progressive disorders (38, 39). These spheroidal inclusions lack limiting membranes, the inclusions are positive for partially degraded neurofilament triplet proteins, and in some perikarya they form juxtanuclear complexes comparable in size to the links induced by truncated desmin in PtK2 cells. (*iv*) Hirano bodies are a class of non-membrane-bound protein complexes found in normal individuals as well as in patients with amyotrophic lateral sclerosis or with Alzheimer disease (40, 41). These variably sized inclusions consist of paracrystalline structures that stain positively for cytoskeletal proteins including actin, tropomyosin, α-actinin, vinculin, and tau. (*v*) An abnormal conformation of the ubiquitous prion protein can lead to a class of spongiform neurodegenerative diseases (42, 43). Expression or introduction of an altered prion protein can result in a degenerative conversion of the endogenous prion molecules in a chain reaction that produces fibrillar amyloid precipitates, leading ultimately to cell death.

The similarities between these naturally occurring pathologies and the artificially produced syndrome described here suggest that a similar protein-precipitation mechanism may underlie many known disease conditions. In all these cases, it appears that an uncontrolled association and precipitation process is generated initially by the introduction of an aberrant molecule capable of binding endogenous proteins in the normal way at some sites but also in an altered way at others. In the classical sickle hemoglobin (HbS) system, the geometry of the hemoglobin molecules and their association sites cause the HbS precipitate to grow in a regular, unidirectional way (44) and to entrain non-HbS-hemoglobins (45). The precipitates described above incorporate protein molecules

in an irregular geometry that causes them to grow nondirectionally and thus produce an unbounded, insoluble, three-dimensional complex. They all grow by diverting endogenous proteins involved in the formation and/or turnover of normal cellular structures and perhaps by trapping incompletely folded proteins during their folding process. All these precipitates are also similar in their ability to escape the normal mechanisms for prompt degradation of altered intracellular proteins. *In vivo*, if a cell should die following the assembly of a chimeric link, it is probable that the link would persist for some time as a plaque. Alternatively, if *in vivo* a cell which had assembled a desmin/vimentin bolus underwent lysis, that insoluble structure would probably persist for some time as a tangle.

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