Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 754–757, February 1982
Biochemistry

In vitro reconstitution of intermediate filaments from mammalian neurofilament triplet polypeptides

(cytoskeleton/neuronal architecture)

ROBERT V. ZACKROFF*†, WILLIAM W. IDLER‡, PETER M. STEINERT†, AND ROBERT D. GOLDMAN*†

*Department of Biological Sciences, Carnegie-Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213; and ‡Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Communicated by Alex B. Novikoff, October 9, 1981

ABSTRACT **Intermediate filaments (IF) were reconstituted *in vitro* from bovine neurofilament triplet polypeptides. Neural IF, solubilized in either low salt or 8 M urea solution, assembled into IF when returned to near-physiological solution conditions. The 68,000-dalton component of the triplet, purified to homogeneity by preparative $NaDodSO_4$ electrophoresis, was renatured and reassembled into short (≈0.05-μm) ≈10-nm-diameter filaments. These results demonstrate that the triplet polypeptides are components of neural IF and that the 68,000-dalton polypeptide is an IF structural protein.**

Intermediate filaments (IF) are major cytoskeletal elements of nerve axons. The initial identification of specific polypeptides as the structural building blocks of an IF system was provided by the *in vitro* reconstitution of IF from bovine epidermal keratin proteins (1, 2). Several other types of IF have since been reconstituted *in vitro* and their major structural polypeptides thereby identified (3–7). The polypeptides comprising IF are extremely heterogeneous, and IF from different types of cells exhibit marked differences in their size, antigenicity, and conditions for *in vitro* assembly (1, 2). Many of these polypeptides have been extensively studied and, in all instances, have been shown to possess a common α-helical structure (8, 9).

Mammalian neural IF are thought to contain the so-called "neurofilament triplet" polypeptides. These three proteins have molecular masses of ≈200, ≈150, and ≈68 kilodaltons (kDal). They are found in preparations of isolated mammalian neural IF (9–12). Although their molecular masses differ slightly in different mammalian species, peptide mapping studies have shown a high degree of similarity when each molecular mass class is compared across species lines (13). However, the identity of these proteins as neural IF subunits has rested on circumstantial evidence, because IF have not yet been reassembled from these proteins *in vitro*.

In this report, we show that the triplet proteins copurify with *in vitro*-reassembled neural IF. These IF are ≈10 nm in diameter and are morphologically indistinguishable from freshly isolated neural IF. At least one of the triplet polypeptides (68 kDal) is a neural IF structural protein, as it can assemble into short ≈10-nm-diameter filaments in the absence of the 150,000- and 200,000-dalton polypeptides.

MATERIALS AND METHODS

Neural IF were isolated from bovine spinal cord within 90 min of slaughter, using a modification of the technique introduced by Delacourte *et al.* (14). Spinal cords were homogenized in isolation buffer [0.1 M 4-morpholineethanesulfonic acid (Mes), pH 6.6/5 mM EGTA/0.5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$)]. For each g (wet weight) of tissue, 1.7 ml of isolation buffer was added. The homogenate was clarified by centrifugation (12,500 rpm, 20 min, GSA rotor, 0°C), and the supernatant was further purified by high-speed centrifugation (78,000 × *g*, 30 min, 0°C). The resulting supernatant was made 20% in glycerol, warmed briefly to 37°C (≈5 min) and centrifuged again (78,000 × *g*, 45 min, 30°C). The pellets were suspended in isolation buffer, homogenized, and centrifuged (105,000 × *g*, 30 min, 0°C). The resulting pellets consisted of a loose upper layer and a compact lower layer. The loose upper layer contained IF as determined by negative staining. These IF were recovered by gently swirling the tubes and stored at −80°C until used. The supernatant was then made 20% in glycerol, warmed to 37°C, and centrifuged (105,000 × *g*, 30 min, 30°C). These pellets constituted a second source of IF and were indistinguishable on $NaDodSO_4$ gels from the loose pellet. The two IF fractions were used interchangeably in all experiments and yielded identical results. Pellets containing IF could be stored for later use at −80°C.

Solubilization and Reconstitution of Neural IF. Preparations of isolated IF were dissolved by homogenization (glass/Teflon) in urea disassembly buffer (8 M urea/20 mM Mes, pH 6.6/1 mM EGTA/1 mM $PheMeSO_2F$/0.1 mM $MgCl_2$/1% 2-mercaptoethanol) (3–5 mg of protein/ml of buffer). These preparations were centrifuged at 250,000 × *g* for 1 hr at 15°C. The supernatants were recovered and dialyzed for 12 hr at 25°C against 1000 vol of assembly buffer (0.15 M KCl/25 mM imidizole·HCl, pH 7.1/5 mM $MgSO_4$/2 mM dithiothreitol/0.125 mM EGTA/0.2 mM $PhMeSO_2F$). The resulting reassembled IF were harvested by centrifugation (150,000 × *g*, 45 min, 25°C). The pellets were dissolved in disassembly buffer (1 to 2 mg/ml of protein) and further purified by repeated cycles of the above procedure.

In an alternative procedure, neural IF disassembly was obtained by dialysis at 4°C of freshly isolated IF (24 hr with two buffer changes) against 1000 vol of TEBP buffer (5 mM Tris/0.125 mM EGTA/0.1% 2-mercaptoethanol/0.1 mM $PhMeSO_2F$, pH 8.6, adjusted with NaOH). These preparations were clarified by centrifugation (250,000 × *g*, 90 min, 0°C). Assembly of IF was induced by addition of 0.1 vol of 1.5 M KCl/0.25 M imidazole·HCl, pH 6.05/50 mM $MgSO_4$, which resulted in a final pH of 6.8–7.0. Reassembly of IF was allowed to proceed for 1 hr at 37°C. The reassembled IF were then harvested by centrifugation as described above.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: IF, intermediate filament(s); kDal, kilodalton(s); Mes, 4-morpholineethanesulfonic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

† Present address: Department of Cell Biology and Anatomy, Northwestern University, Chicago, Illinois 60611.

Analytical Procedures. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out on 7.5% acrylamide slab gels with 4.5% stacking gels according to the procedure of Laemmli (15). Electrophoresis in tube gels (7% acrylamide/3% bisacrylamide) was carried out using 0.5% $NaDodSO_4$ in the upper electrode buffer as described (9). Preparative electrophoresis was performed on an LKB Uniphor using 7% T for elution of the 68-kDal polypeptide and 4% T for elution of the 160 and 200-kDal polypeptides (9). $NaDodSO_4$ was removed from the purified polypeptides by ion pair extraction (16), and the polypeptides were suspended in urea disassembly buffer and dialyzed into assembly buffer.

Protein was estimated by the method of Bradford *et al.* (17) using bovine serum albumin as standard. Negative staining with 1.5% uranyl acetate was performed after fixing the protein with 1% glutaraldehyde. Preparations containing 8 M urea were glutaraldehyde-fixed and then diluted with water to a final urea concentration of <0.5 M prior to negative staining. Samples were observed and photographed using a Philips 201C electron microscope operated at 60 kV.

RESULTS

Conditions for Disassembly of Native Neural IF. Isolated neural IF at 0.8–1.2 mg/ml were dissociated by dialysis against TEBP buffer for 24 hr at 4°C. This procedure resulted in 40–60% solubilization as determined by ultracentrifugation at 250,000 × *g* for 90 min. Negative staining of the supernatants showed the absence of IF and the presence of 3- to 6-mm-diameter protofilamentous subunits of appearance similar to those previously described using other IF systems (3, 5, 7).

Solubilization using 8 M urea was much more efficient than solubilization using low salt; 95–98% of the protein (concentration, ≤ 6 mg/ml) was consistently recovered in the supernatant after centrifugation for 1 hr at 250,000 × *g*. Negative staining of these urea-solubilized preparations showed that they contained very fine filamentous structures, ≈2 nm in diameter and 30–50 nm long. Some other filamentous as well as globular aggregates were seen, but no intact IF were observed in these preparations.

Reassembly of Neural IF from Solubilized Components. Solution conditions for neural IF assembly were determined by examining the effects of pH, monovalent and divalent cation concentrations, and several different buffer systems. In this fashion, a standard assembly buffer (25 mM imidizole·HCl/0.15 M KCl/5 mM $MgSO_4$) was established as optimal. At pH values <6.2 and >7.2, very short (≈0.1 μm) IF were obtained. The longest IF (0.5–1.0 μm) were obtained at pH 6.8–7.1 (Fig. 1). Ca^{2+} was not used because of the possibility of proteolysis due to Ca^{2+}-activated proteases (18) and therefore 0.125 mM EGTA was generally included in the standard assembly buffer. Optimal assembly of IF occurred at 37°C for 1 hr. In assembly buffer (pH 7.1), fewer and much shorter (<0.1 μm) IF were observed by negative staining after assembly at 0°C, even after prolonged (12 hr) incubation. A sulfhydryl reductant (2 mM dithiothreitol or 0.1% 2-mercaptoethanol) was routinely included in the assembly buffer because of its ability to enhance IF assembly in other systems (1, 4, 8). ATP and GTP at 1–5 mM did not affect IF assembly.

The method of solubilization (low salt or urea) did not appear to affect the optimal conditions for reassembly. In appearance in negative stain, the reassembled IF were similar to freshly isolated IF often possessing rough edges (Fig. 1). However, reassembled IF were never as long as freshly isolated IF: the longest reassembled IF were ≈1 μm, compared with ≈5 μm or more in freshly isolated preparations.

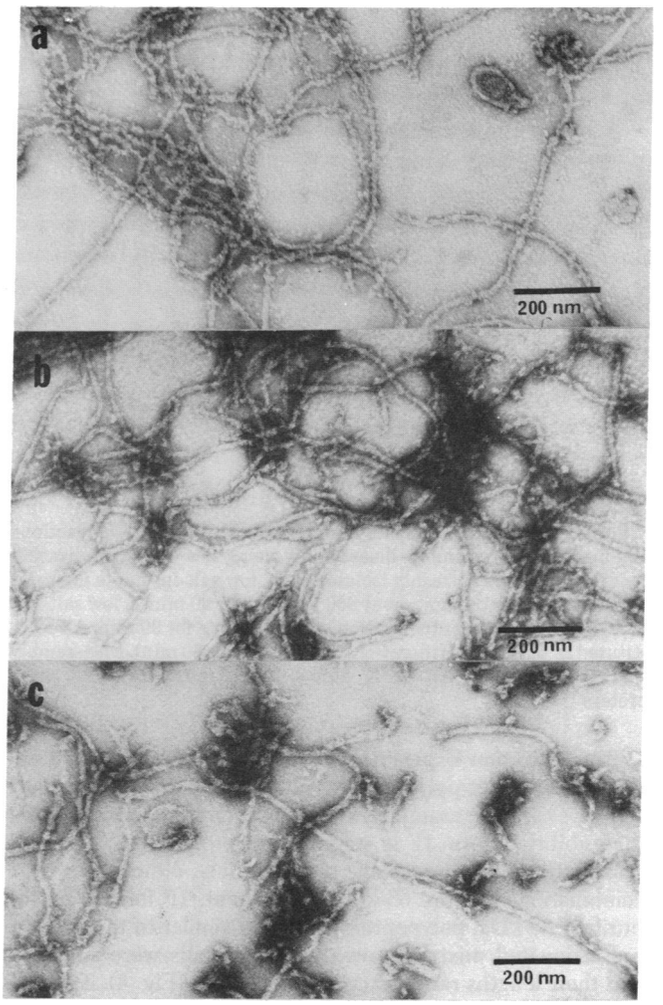

FIG. 1. Negatively stained preparations of freshly isolated neural IF (*a*) and *in vitro*-reconstituted neural IF (*b* and *c*). IF in *b* were reconstituted after disassembly in low salt solution and those in *c* were reassembled after urea solubilization. (×62,000.)

Assembly–Disassembly Cycles. Neural IF preparations isolated according to the Delacourte *et al.* (14) procedure contained, in addition to the 200-, 150-, and 68-kDal triplet components, a variety of other polypeptides in minor amounts (Figs. 2 and 3). The major non-IF contaminant consisted of material in the 55-kDal region, some of which may represent tubulin (14). Assembly–disassembly, using low salt for solubilization, did not greatly change the polypeptide composition of reassembled IF, although the amount of ≈55-kDal material was slightly diminished (Fig. 2). Assembly–disassembly using urea for solubilization resulted in enrichment of the ≈55-kDal material, as well as many other minor components in the urea-insoluble pellets (Fig. 3). However, most of the ≈55-kDal material was retained in IF preparations carried through three assembly–disassembly cycles using urea. These preparations contained the following polypeptides: 200 kDal, 25–30%; 150 kDal, 15–20%; 68-kDal, 15–20%; ≈55-kDal, ≈10%; and minor components, ≈20%. Therefore, *in vitro*-reconstituted IF contained predominantly the triplet polypeptides. The 200- and 150-kDal species were sometimes resolved as doublets. A band that comigrated with microtubule-associated protein was also visible on heavily loaded gels in each of the above preparations (Fig. 2). Since glial IF protein migrates very close to β-tubulin (14), we cannot rule out its presence in small amounts (≤10%).

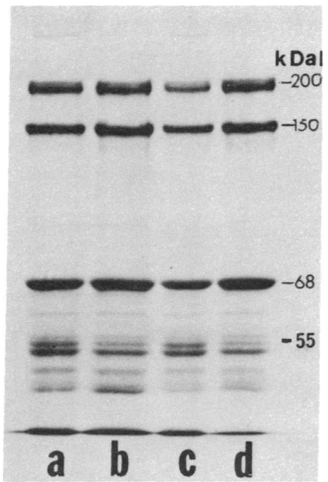

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions obtained during assembly–disassembly using low salt solubilization conditions. Lanes: a, freshly isolated IF; b, low salt-insoluble material in pellet after centrifugation at 250,000 × *g* for 90 min; c, low salt-soluble material after centrifugation at 250,000 × *g* for 90 min; d, reconstituted IF pellet (centrifugation, 150,000 × *g* for 30 min). Disassembly was by dialysis against TEBP buffer. Each lane contains 20 μg of protein.

Reconstitution Experiments with Purified Triplet Polypeptides. The triplet polypeptides, obtained by preparative electrophoresis, were examined individually and in combination for their ability to form IF in standard assembly buffer. The only polypeptide that was capable of reassembly, either alone or in combination, was the 68-kDal component. IF formed by the purified 68-kDal polypeptide appeared similar to those in the unfractionated mixture; however, their walls were smoother and their lengths rarely exceeded ≈0.2 μm (Fig. 4). The 150- and 200-kDal components did not form 10-nm-diameter filaments, possibly because of irreversible denaturation during the preparative procedure.

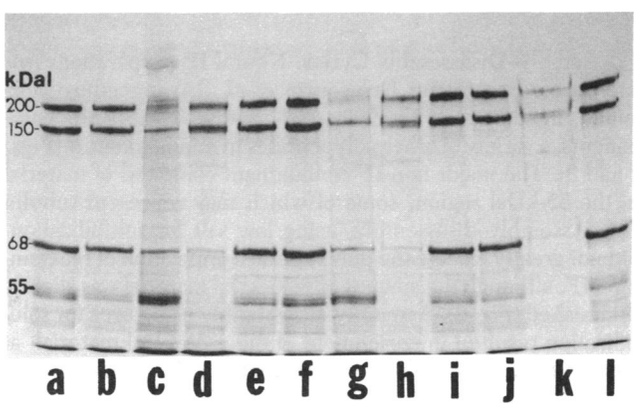

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions obtained during assembly–disassembly using urea for disassembly. Lanes: a, freshly isolated IF; b, supernatant from first 250,000 × *g* clarification; c, pellet from first 250,000 × *g* clarification; d, supernatant remaining after harvesting once-reassembled IF; e, pellet of once-reassembled IF; f, supernatant from second 250,000 × *g* clarification; g, pellet from second 250,000 × *g* clarification; h, supernatant remaining after harvesting twice-reassembled IF; i, pellet of twice-reassembled IF; j, supernatant from third 250,000 × *g* clarification; k, supernatant remaining after harvesting three-times-reassembled IF; l, pellet of three-times-reassembled IF. Each lane contained 10–15 μg of protein, except lanes h and k, which contained 3–5 μg of protein.

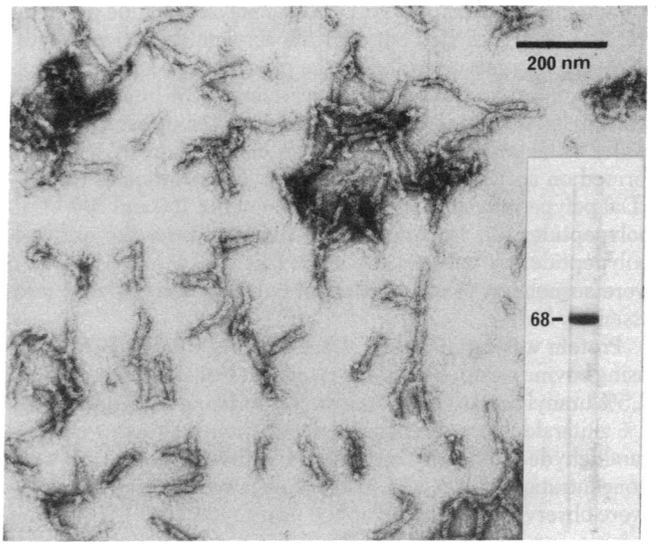

FIG. 4. Negatively stained preparation of short IF obtained from electrophoretically purified 68-kDal polypeptide. (×62,000.) (*Inset*) $NaDodSO_4$/polyacrylamide gel electrophoresis of electrophoretically purified 68-kDal polypeptide. The faint bands below the 68-kDal band are the result of proteolysis of the 68-kDal polypeptide, since they increase during storage and do not comigrate with polypeptides in the unfractionated mixture. IF were reassembled in standard assembly buffer using protein at 0.8 mg/ml.

DISCUSSION

IF from a variety of cells and tissues have been reassembled *in vitro* and their constituent polypeptides have thereby been identified. These systems include epidermal keratin IF, composed of up to 10 polypeptides ranging in molecular mass from 45 to 65 kDal (1, 2); glial IF, composed of a single 51-kDal polypeptide (4); BHK-21 fibroblast IF, composed of two ≈54- and one 55-kDal polypeptides (3); smooth muscle IF, composed of a major 54-kDal polypeptide (6); squid neural IF, composed of polypeptides of 60, 74, 100, and 220 kDal (5); and *Myxicola* neural IF, composed of two polypeptides of 150–160 kDal (7). Although reports of reassembly of IF from mammalian neural tissue have also appeared (19), it is now clear that this protein may have been derived from contaminating glial cells (8, 10–12). Here we have described the successful solubilization and reconstitution of IF from the neural IF triplet polypeptides. Evidence now exists that indicates that these polypeptides are present only in neurons and not in glial or other neuron-associated cells (8, 10–12). From the present studies, we conclude that the triplet polypeptides are indeed the major components of at least one type of neural IF. We do not yet know whether each of the triplet components is associated with different single IF or whether they exist in combination in the same IF as heteropolymers. The 68-kDal polypeptide is, by itself, capable of reassembly into short homopolymer IF; i.e., it is an integral IF wall protein and as such can be considered an IF subunit protein.

It is not clear whether the inability to achieve greater elongation of pure 68-kDal IF is due to a requirement for the higher molecular mass (or other) proteins. It is possible that the homopolymer 68-kDal IF are short due to the fact that the protein becomes denatured during purification or that the concentration of protein is too low. Further work will be required to elucidate the roles of the 150- and 200-kDal polypeptides in the assembly reaction to determine whether these components are IF structural proteins, accessory proteins, or possible elongation factors. Recent data using antibody-labeling techniques

agree with the present studies and suggest that the 68-kDal component is an IF subunit protein (20). These data also suggest that the 150- and 200-kDal components are closely associated with, but are not integral structural components of, neural IF (20). It should be noted that pure 68-kDal IF appear smooth walled and therefore resemble IF reassembled from the majority of other IF systems studies so far (1–7). On the other hand, IF reassembled from unfractionated triplet polypeptides often appear rough walled. It is possible, therefore, that the 150- and 200-kDal polypeptides are responsible for the rough-walled appearance of mammalian neural IF. Further reconstitution studies are required to investigate this possibility.

The ability to reassemble neural IF *in vitro* should enable further studies on the assembly mechanism and ultimately may help to elucidate directly the possible roles of each of the triplet polypeptides in neural IF assembly and function.

We thank the National Science Foundation and the National Institutes of Health for their generous support. R.V.Z. is the recipient of a National Institutes of Health Postdoctoral Fellowship.

1. Steinert, P. M., Idler, W. & Zimmerman, S. B. (1976) *J. Mol. Biol.* **108,** 547–567.
2. Steinert, P. M. & Gullino, M. I. (1976) *Biochem. Biophys. Res. Commun.* **70,** 221–227.
3. Zackroff, R. V. & Goldman, R. D. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6226–6230.
4. Reuger, D. C., Huston, J. S., Dahl, D. & Bignami, A. (1979) *J. Mol. Biol.* **135,** 53–68.
5. Zackroff, R. V. & Goldman, R. D. (1980) *Science* **208,** 1152–1155.
6. Huiatt, T. W., Robson, R. M., Arakawa, N. & Stromer, M. H. (1980) *J. Biol. Chem.* **255,** 6981–6989.
7. Gilbert, D. S., Newby, B. J. & Anderton, B. H. (1975) *Nature (London)* **256,** 586–589.
8. Zackroff, R. V., Steinert, P., Whitman Aynardi, M. & Goldman, R. D. (1981) in *Cell Surface Reviews,* eds. Nicolson, G. & Poste, G., In press.
9. Steinert, P. M., Idler, W. W. & Goldman, R. D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4534–4538.
10. Liem, R. K. H., Yen, S.-H., Salomon, G. D. & Shelanski, M. L. (1978) *J. Cell. Biol.* **79,** 637–645.
11. Schlaepfer, W. W. & Freeman, L. A. (1978) *J. Cell Biol.* **78,** 653–662.
12. Shelanski, M. L. & Liem, R. K. H. (1979) *J. Neurochem.* **33,** 5–13.
13. Chiu, F.-C., Korey, B. & Norton, W. T. (1980) *J. Neurochem.* **34,** 1149–1159.
14. Delacourte, A., Fillatreau, G., Boutteau, F., Biserte, G. & Schrevel, J. (1980) *Biochem. J.* **191,** 543–546.
15. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
16. Henderson, L. E., Oroszlan, S. & Konigsberg, W. (1979) *Anal. Biochem.* **93,** 153–157.
17. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
18. Schlaepfer, W. W. & Micko, S. (1979) *J. Neurochem.* **32,** 211–219.
19. Huneeus, F. C. & Davison, P. F. (1970) *J. Mol. Biol.* **52,** 415–428.
20. Willard, W. & Simon, C. (1981) *J. Cell Biol.* **89,** 198–205.