NF-L and NF-M are expressed in epithelial cells and coassemble with keratin or vimentin

PAN Ning, SUN Yuhui, CHEN Shuzhen, JIN Yang & CHEN Jianguo

College of Life Science, Peking University, Beijing100871, China
Correspondence should be addressed to Chen Jianguo (e-mail: chenjg@pku.edu.cn)

Abstract  Neurofilaments (NFs) and cytokeratins are both heteropolymers, which assemble into intermediate filaments (IFs) only when other proper IF subunit proteins are expressed simultaneously. To study the assembly property of NFs, we constructed two recombinant adenovirus which could express NF-L or NF-M, fused with green fluorescent protein (GFP) respectively. Then they were introduced into vero cells, and expressed fusion protein. Double labels of GFP fluorescence and immunofluorescence staining indicated that NF-L-GFP or GFP-NF-M not only coassembled with endogenous vimentins, but also coassembled with keratins into a cytoplasmic network of filaments.

Keywords: NF-L, NF-M, keratin, vimentin, vero cell.

Among the three main cytoskeletal components of eukaryotic cells, the subunits from which microtubules and microfilaments are assembled have highly conserved amino acid sequences. However, neurofilaments (IFs) are composed of different types of proteins in different tissues and in the cells of different differentiation stages. Usually, types I and II IF proteins, acidic and neutral-basic keratins, are only expressed in the early embryonic cells of early phase and epithelial cells. Type III vimentins are expressed in mesenchyme derived cells and cultured cells. Type IV neurofilaments are the primary cytoskeletal components of neuronal cells[1]. Mammalian neurofilaments are composed of three protein subunits called NF-L, NF-M and NF-H with apparent molecular masses of 68, 150 and 200 ku, respectively[2-4].

During the mammalian embryonic development, keratins are the earliest expressed IF proteins[5]. In the stem cells which will differentiate into neurons, the components of IF networks change from keratins to vimentins. Up to later embryogenesis, the vimentin system is replaced by NF-L and NF-M. Finally, NF-H is also incorporated into the neurofilament networks[6, 7].

Unlike type III IF proteins, keratins and neurofilaments are heteropolymers. In epithelial cells, type I acidic keratins and type II neutral-basic keratins are always coexpressed and coassemble into 10-nm filaments[8]. Some cultured epithelial cells express keratins and vimentins simultaneously as a consequence of adaptation to tissue culture, but they assemble into two distinct filament networks[9]. Coexpression of NF-L with either NF-M or NF-H is necessary for the formation of neuron-specific filamentous structures[10-13], although NF-L can self-assemble to 10-nm filaments. It has been shown that each of the three types of neurofilament proteins can coassemble with endogenous vimentins when it is transfected into fibroblast cells[14,15].

In some neuroendocrine tumor cells with epithelial origin, such as in PC12 cells, cytokeratin and NF proteins are often coexpressed and can form distinct IF network independently. For different types of IF proteins, why can some coassemble into 10-nm filaments, while others do it alone? Apparently, it depends on different primary structures of polypeptide chains. If we get better understanding of the interaction among cytoskeletal proteins, we can get better comprehension of the assembly of cell structures. But first of all, we must know which types of IF proteins can coassemble. In our experiments, we transfected NF-L-GFP or GFP-NF-M into vero cells which could express endogenous keratins, and found that each of the two neurofilament proteins can coassemble with endogenous keratins or vimentins to form a filamentous structure.

1 Materials and methods

(i) Construction of adenovirus transfection vector. The cDNA encoding NF-L or NF-M was fused with the reported gene EGFP. The constructs were cloned to cosmid vector pAxCAwt[16], then 293 cells were cotransfected with Ad5 dix DNA-TPC. After 24 h, the 293 cells were transplanted to a 96-well plate and cultured for over 2 weeks. Then the recombinant viruses were sifted, which could express the fused proteins in cells.

(ii) Cell culture and infection of adenovirus. The 293 cells and vero cells were both cultured in DMEM medium (GIBCO BRL) containing 10% FBS at 37°C under 5% CO2. Cells for immunofluorescence were grown on sterile coverslips. 1 h after the infection of recombinant virus, cells were cultured with normal medium for 48 h. The cultured cells for immunoblot analysis were infected, and then the viruses were harvested 48 h after infection.

(iii) Immunoblot analysis. Infected cells were washed with PBS and subsequently solubilized in SDS-sample buffer. After being boiled for 10 min, the proteins were separated by electrophoresis on 10% polyacrylamide gels and were electrotransferred onto nitrocellulose membranes. The membranes were first incubated with mouse monoclonal anti-NF-L (NF4, Sigma), anti-NF-M (NN18, Sigma), anti-vimentin (V9, Sigma), and anti-keratin antibodies (AE1, reacts with most acidic keratins; AE3, a gift from Prof. T.T. Sun in New York University, reacts with neutral-basic keratins), respectively. The blots were then incubated with alkaline phosphatase-
conjugated goat anti-mouse IgG. The specific protein bands were visualized with BCIP and NBT.

(iv) Immunofluorescence staining. Infected cells were fixed with freshly prepared 4% polyformalin in PBS for 15 min at room temperature. After being incubated with either monoclonal anti-vimentin or anti-keratin antibodies, the cells were incubated with rhodamine-conjugated goat anti-mouse IgG. The cells were subsequently washed and mounted onto slides with 50% glycerol in PBS, and then observed under a fluorescence microscope.

2 Results

(i) Vero cells expressing keratin and vimentin simultaneously. Vero cells belong to epithelial cells, in which acidic keratins and neutral-basic keratins are coexpressed and coassemble into 10-nm filaments. However, in vitro the cytoplasmic intermediate filaments often change from one type to another. In some epithelial cells, besides the acidic and basic keratins, vimentins are simultaneously expressed, and they form two different IF systems\[9\]. To identify the types of endogenous IF proteins, the cultured vero cells were washed with PBS and solubilized in SDS-sample buffer. Immunoblotting analysis showed that besides the 40 and 52 ku keratins, a 55 ku vimentin was also expressed in vero cells (fig. 1(a)).

(ii) Construction of recombinant adenosine virus. To examine if neurofilament proteins were expressed, vero cells were infected with recombinant adenosine containing NF-L-GFP or GFP-NF-M cDNA. Immunoblotting analysis showed that a 95 ku fusion protein was expressed in the cells infected with NF-L-GFP cDNA, and a 177 ku fusion protein was expressed in the cells infected with GFP-NF-M cDNA (fig. 1(b)).

(iii) Coassembly of NF-L and NF-M with keratin or vimentin to form filamentous networks. To investigate if the assembly properties of neurofilament proteins were affected by fusing to GFP, transfected vero cells were observed using 488 nm light for stimulating. Filamentous structure could be seen in the cells (Plate I -2, 4, 6, 8), which showed that fusing GFP to the C-terminus of NF-L (Plate I -2, 4) or to the N-terminus of NF-M (Plate I -6, 8) did not affect the assembly of neurofilament proteins into intermediate filaments.

Since two types of IF proteins, vimentin and keratins, were present in vero cells, to determine with which type neurofilament proteins could coassemble, the transfected cells were incubated with either anti-vimentin or anti-keratin antibodies, and subsequently stained with rhodamine-conjugated goat anti mouse IgG. The GFP showed the location of neurofilament proteins, while rhodamine showed the location of keratins or vimentin. The result indicated that in vero cells not only could NF-L and NF-M incorporate into endogenous vimentin filament network (Plate I -1, 5) but also could coassemble with keratins to form filamentous structures (Plate I -3, 4).

3 Discussion

Intermediate filaments are tissue-specific cytoskeletal proteins. Different types of IFs are expressed in different cells or in cells of different developmental stages\[11\]. Vero cells came from the kidney of African Green Monkey and belong to epithelial cells. Immunoblotting analysis showed that a 40 ku protein recognized by monoclonal antibody AE1 and a 52 ku protein recognized by monoclonal antibody AE3 were present in vero cells. AE1 can recognize most acidic keratins. The 40 ku protein may correspond to K19. AE3 can recognize almost all neutral-basic keratins. So far, we cannot determine which type the 52 ku keratin is. The presence of the 55 ku vimentin in vero cells possibly results from the change of the circumstance of the cells. When transfected into fibroblast cells which usually express vimentins only, each of the three neurofilament proteins can coassemble with vimentin into 10-nm filaments\[14,15\]. When transfected into sf9 cells which lack endogenous IF proteins, NF-L can self-assemble into loose intermediate-filament-like structure in partial area of the cells, while the assembly of NF-M or NF-H into filaments requires the copresence of NF-L\[16,17\], which indicated that neurofilaments were heteropolymers\[18\]. NF-L plays a dominant role in the assembly of filaments, while NF-M and NF-H contribute to the formation of the structural characteristics of neurofilaments.

During the course of cell differentiation, the transition of intermediate filaments has two different ways: one is that the newly synthesized IF proteins gradually replace the old type by coassembly; the other is that the new IF proteins form a new filament network. In the differentiation of neurons, the transition type probably is the latter, while the replacement of vimentins by neurofilaments is

NOTES

Plate 1. Immunoblotting of the intermediate filaments expressed in vero cells. (a) The endogenous 40 ku acid keratins (1), 52 ku basic keratin (2) and 55 ku vimentin (3) were detected with AE1, AE3, and anti-vimentin antibodies, respectively; (b) immunoblotting of fusion proteins of NF-L-GFP and GFP-NF-M expressed in vero cells. 95 ku NF-L-GFP (1) and 177 ku GFP-NF-M (2) were detected with anti-NF-L and anti-NF-M antibodies, respectively.