Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 337 (2005) 68–74
BBRC
www.elsevier.com/locate/ybbrc

Localization of NGF and TrkA at mitotic apparatus in human glioma cell line U251

Zhijian Zhang, Yong Yang, Aihua Gong, Cunzu Wang, Yan Liang, Yongchang Chen *

School of Medicine, Jiangsu University, Jiangsu Zhenjiang 212001, China

Received 25 August 2005
Available online 13 September 2005

Abstract

It has previously been implicated that nerve growth factor (NGF) with its high-affinity receptor tyrosine kinase A (TrkA) could play an important role in the growth modulation of human tumor cells, such as glioblastoma multiform cell lines and human breast cancer cell lines. However, the direct mitogenic effects of NGF and TrkA in these tumor cells still remain to be elucidated. Herein we show, by immunofluorescence staining, that NGF was colocalized with γ-tubulin at the centrosomes or the spindle poles throughout the cell cycle and phosphorylated TrkA was colocalized with α-tubulin at mitotic spindle in the glioma cell line U251. The results suggest that NGF concentrated to centrosome can recruit its receptor TrkA there and cause phosphorylation of the latter. The phosphorylated TrkA with the tyrosine kinase activity may phosphorylate the tubulin and promote the mitotic spindle assembly. By these mechanisms, NGF can modulate the mitosis of human glioma cells.
© 2005 Elsevier Inc. All rights reserved.

Keywords: NGF; TrkA; Distribution; Centrosome; Spindle

The centrosome is the primary microtubule organizing center and a construction binding a lot of bioactive proteins in mammalian cells. The structure of centrosome is dynamical and remodeled not only during replication in interphase but also at the onset of mitosis, as it acquires many new components involved in generating the spindle pole, assembling mitotic apparatus, and responding to the cell cycle checkpoint [1–6]. Many cell cycle regulatory proteins, including cyclins, CDKs, and p53, were found recruited to the centrosome to elicit proper G1, S, and G2 checkpoint responses [7–11]. A number of protein kinases such as casein kinase [12], polo-like kinase [13], TTK kinase [14], Mpsl protein kinase [15], and PKC [16] have also been localized to the centrosome or spindle. Binding of these proteins to centrosomes causes the local concentration of the proteins to be much higher than it would be if the proteins were dispersed evenly through the nucleoplasm and/or the cytoplasm. This high local concentration facilitates interactions among these proteins (e.g., phosphorylation of substrates by kinases). Growth factors and their receptors are important growth modulators of human tumor cells. Genetic aberration in growth factor mediated signaling pathways is linked to genesis of malignant tumor cells [17–21] Whether growth factors and their receptors are recruited to the centrosome or spindle and involved in the onset of mitosis of tumor cells is unknown. To explore the role of nerve growth factor (NGF) and its high-affinity receptor TrkA in modulating the mitosis of human glioma cells, we have observed the co-localizations of NGF and phosphorylated TrkA with γ-tubulin or α-tubulin at the centrosomes and mitotic apparatus of the human glioma cell line U251 cells by using immunofluorescence staining.

Materials and methods

Cell culture. Human glioma cell line U251 was obtained from Institute of Cell Biology of Chinese Academy of Sciences. The cells were routinely

* Corresponding author.
E-mail address: ycchen54@ujs.edu.cn (Y. Chen).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.265

cultured in DMEM (Gibco) plus 10% fetal bovine serum (Gibco) in a 5% CO_2 incubator at 37 °C. The cells were allowed to grow on 12-mm round glass coverslips in 24-well plates for 72 h.

Immunofluorescence staining. After incubated with 0.2 μM nuclear fluorochrome bisbenzimide (Hoechst 33342, Sigma) for 5 min to reveal nuclei, U251 cells on 12-mm coverslips were fixed in 4% paraformaldehyde

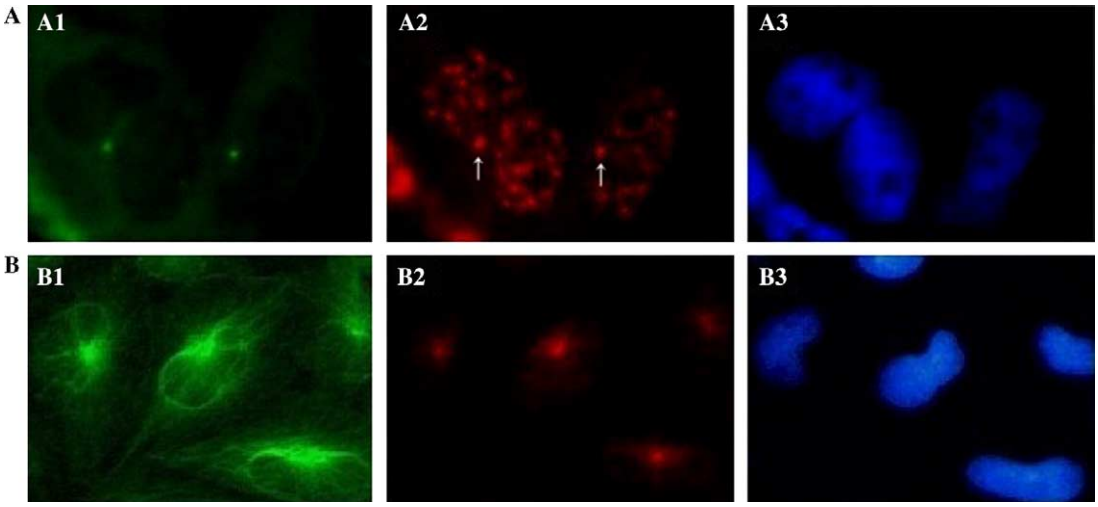

Fig. 1. Distribution of NGF in interphase of U251 cells. (A) After PAF fixation followed by a post-fixation with methanol at −20 °C, the centrosome was detected with monoclonal antibody against γ-tubulin (A1) and polyclonal antibody against NGF (A2, arrows indicate centrosomes). (B) The cytoskeleton was stained by monoclonal antibody against α-tubulin (B1) which irradiated from the NGF staining foci (B2). (A3,B3) Nuclei were stained by Hoechst 33342.

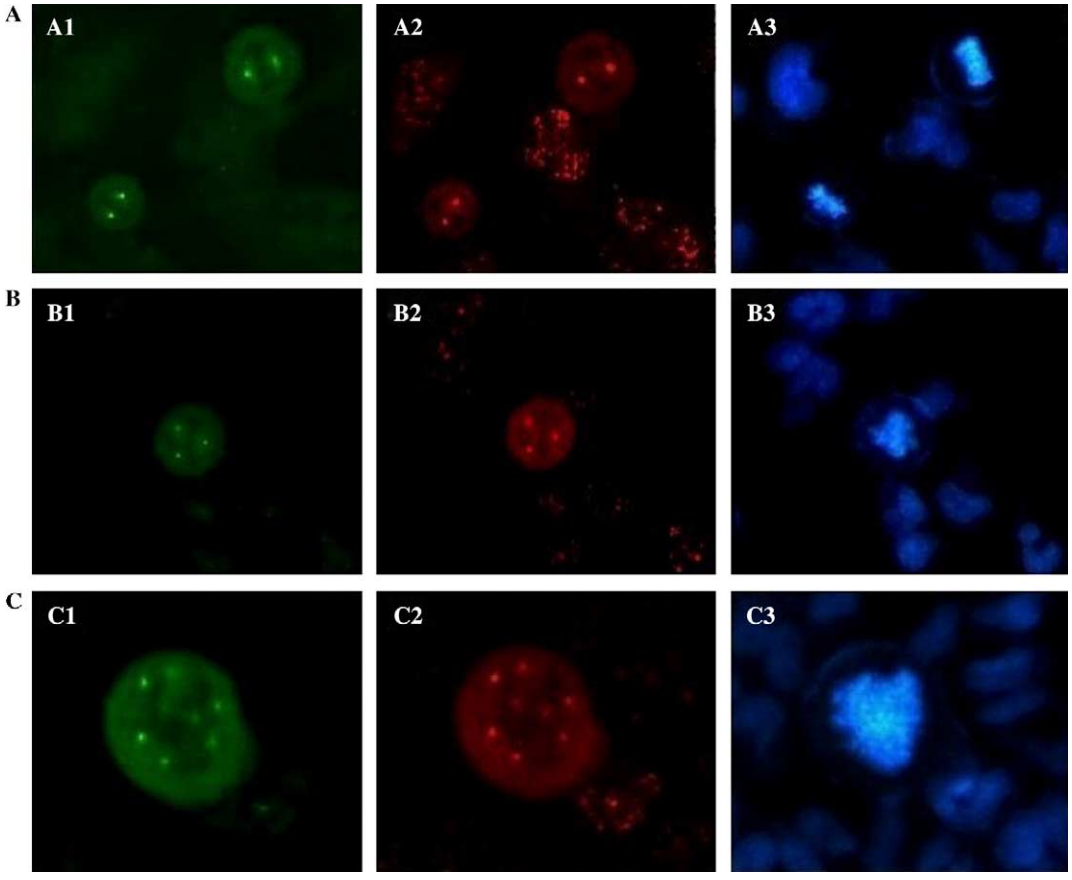

Fig. 2. NGF was colocalized with γ-tubulin at the centrosomes in mitotic cells. (A1, B1, and C1) The centrosomes were detected by monoclonal antibody against γ-tubulin. (A2, B2, and C2) By using polyclonal antibody against NGF, the fluorescent staining was localized at the same sites with γ-tubulin staining. Note that there were more than two centrosomes in the cells (B1, B2, C1, and C2). (A3, B3, and C3) The chromosome was stained by Hoechst 33342.

for 15 min at RT followed by post-fixation with methanol for 6 min at −20 °C. The fixed cells were incubated with 1% BSA in phosphate-buffered saline containing Triton X-100 (PBS-T, 20 mM phosphate buffer, pH 7.5, 150 mM NaCl, and 0.03% Triton X-100). Immunofluorescence co-staining was carried out with the following protocol. The cells were incubated with mouse monoclonal antibody against γ-tubulin (Sigma) or α-tubulin (Sigma) for 2 h at RT. The cells were washed with PBS-T and the primary antibody was visualized by subsequent incubation with the secondary antibody (goat anti-mouse IgG) conjugated with FITC (Sigma). After the first staining, the cells were incubated with rabbit polyclonal antibody against NGF (H-20, Santa Cruz) or phosphorylated TrkA (Tyr490, Santa Cruz). The two antigens were detected with Cy3-conjugated goat anti-rabbit IgG (Sigma). Coverslips were washed with PBS-T and mounted in 90% glycerol/10% Tris, pH 7.5. The fluorescence of Cy3 (red), FITC (green), and Hoechst 33342 (blue) was observed under a fluorescence microscope with a CCD camera (Leica).

Results

Distribution of NGF in interphase of U251 cells

The clear fluorescence staining dots of NGF were revealed at centrosomes in addition to distribution in the nuclei. The immunofluorescence co-staining showed that NGF was colocalized with γ-tubulin at the centrosomes (Fig. 1A). The microtubule bundle stained by α-tubulin was irradiated from the NGF staining foci (Fig. 1B).

NGF was concentrated at the spindle poles of mitotic cells

The immunofluorescence co-staining showed that NGF was colocalized with γ-tubulin to the spindle poles of mitotic cells (Fig. 2), from which the spindles stained by α-tubulin were irradiated to chromosome which was stained with Hoechst 33342. However, the chromosome is negative for NGF staining (Fig. 3). In some mitotic cells, more than two co-staining dots (centrosome number amplification) were observed (Figs. 2B and C, and 3B and C).

Distribution of phosphorylated TrkA in interphase of U251 cells

Immunofluorescence staining revealed that the phosphorylated TrkA was localized on the membrane and the processes of the interphase U251 cells. The cytoplasm and nucleus were stained weakly. The cytoskeleton was not revealed (Fig. 4).

The phosphorylated TrkA was localized on mitotic spindle

The phases of mitotic cells were differentiated by chromosome staining with Hoechst 33342. Immunofluorescence co-staining showed that the phosphorylated TrkA was

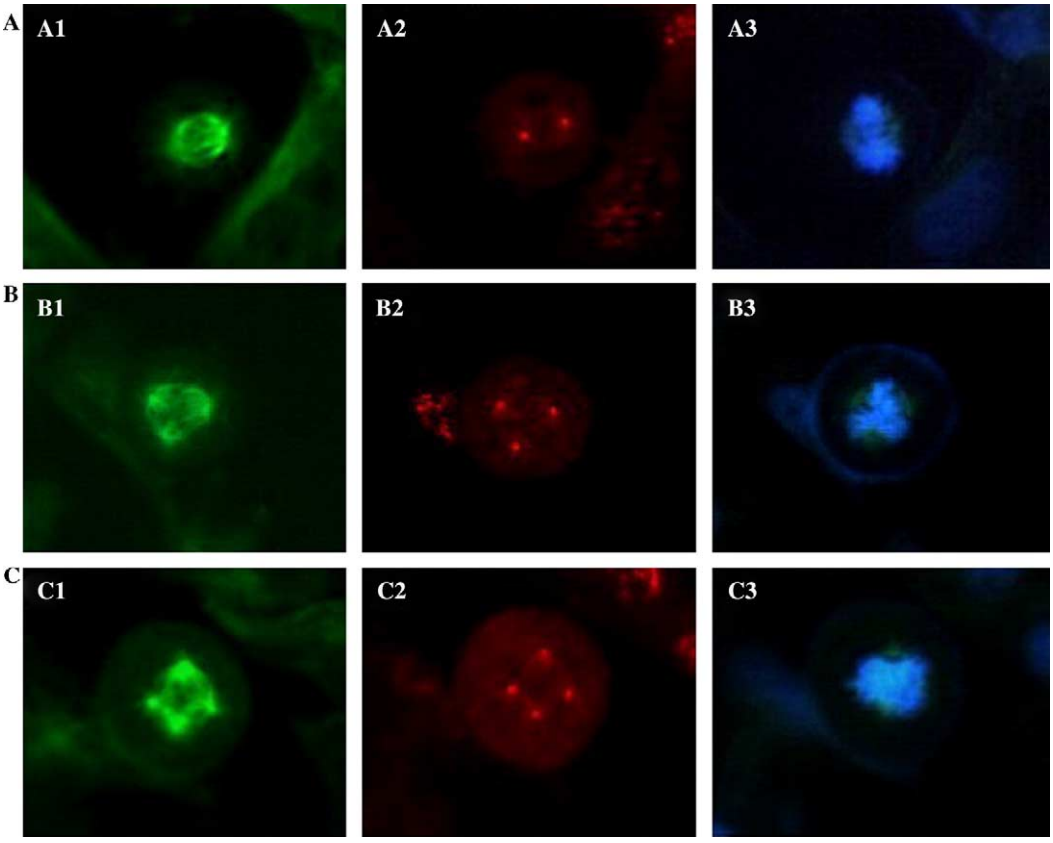

Fig. 3. NGF was anchored at the spindle poles of mitotic cells. (A1, B1, and C1) The mitotic spindles were stained by monoclonal antibody against α-tubulin. (A2, B2, and C2) Immunofluorescence staining with polyclonal antibody against NGF showed that NGF was anchored at the spindle poles of mitotic cells. Note that the multipolar (tripolar, tetrapolar) spindles were revealed in the mitotic cells (B1,C1). (A3, B3, and C3) The chromosome was stained by Hoechst 33342.

colocalized with α-tubulin at the mitotic spindle from prophase to anaphase of the mitotic cells. From metaphase to late anaphase the staining became weaker (Figs. 5A–D). In telophase, as the mitotic spindle disappeared, the dim co-staining was at the midbody (Fig. 5E). In addition to localization at the normal bipolar spindle, the phosphorylated TrkA was also localized at the multipolar (tripolar, tetrapolar, and so on) spindles (Fig. 6).

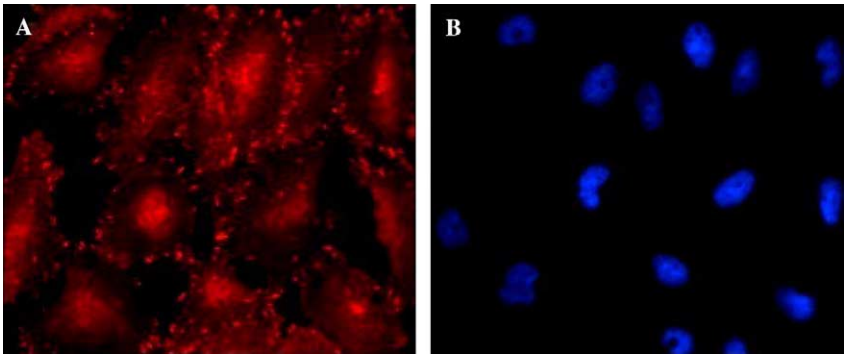

Fig. 4. Distribution of phosphorylated TrkA in interphase of U251 cells. (A) Immunolocalization of phosphorylated TrkA in the cells by using polyclonal antibody against phosphorylated TrkA after PAF fixation followed by cold methanol post-fixation. Immunofluorescence staining was localized on the membrane. (B) The nuclei of interphase were visualized by Hoechst 33342.

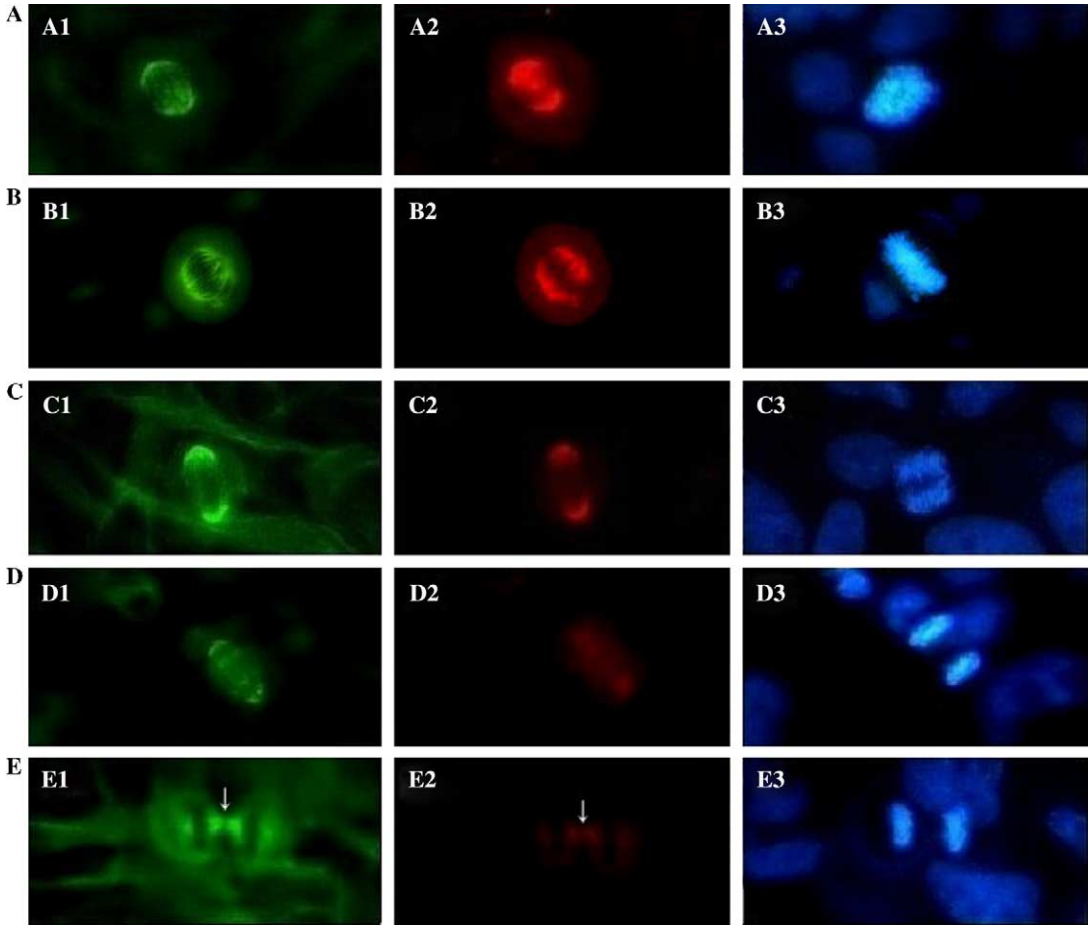

Fig. 5. The phosphorylated TrkA was colocalized with α-tubulin on mitotic spindle. The mitotic spindles (A1, B1, C1, and D1) and midbody (E1, the arrow indicated) were stained by monoclonal antibody against α-tubulin. By using polyclonal antibody against phosphorylated TrkA, the immunofluorescence staining was localized on the mitotic spindle from prophase to anaphase of the mitotic cells (A2, B2, C2, and D2). From metaphase to late anaphase the staining became weaker. In telophase, the weak staining was dim at the midbody (E2, the arrow indicated). The prophase (A3), metaphase (B3), anaphase (C3), late anaphase (D3), and telophase (E3) were defined by chromosome staining with Hoechst 33342.

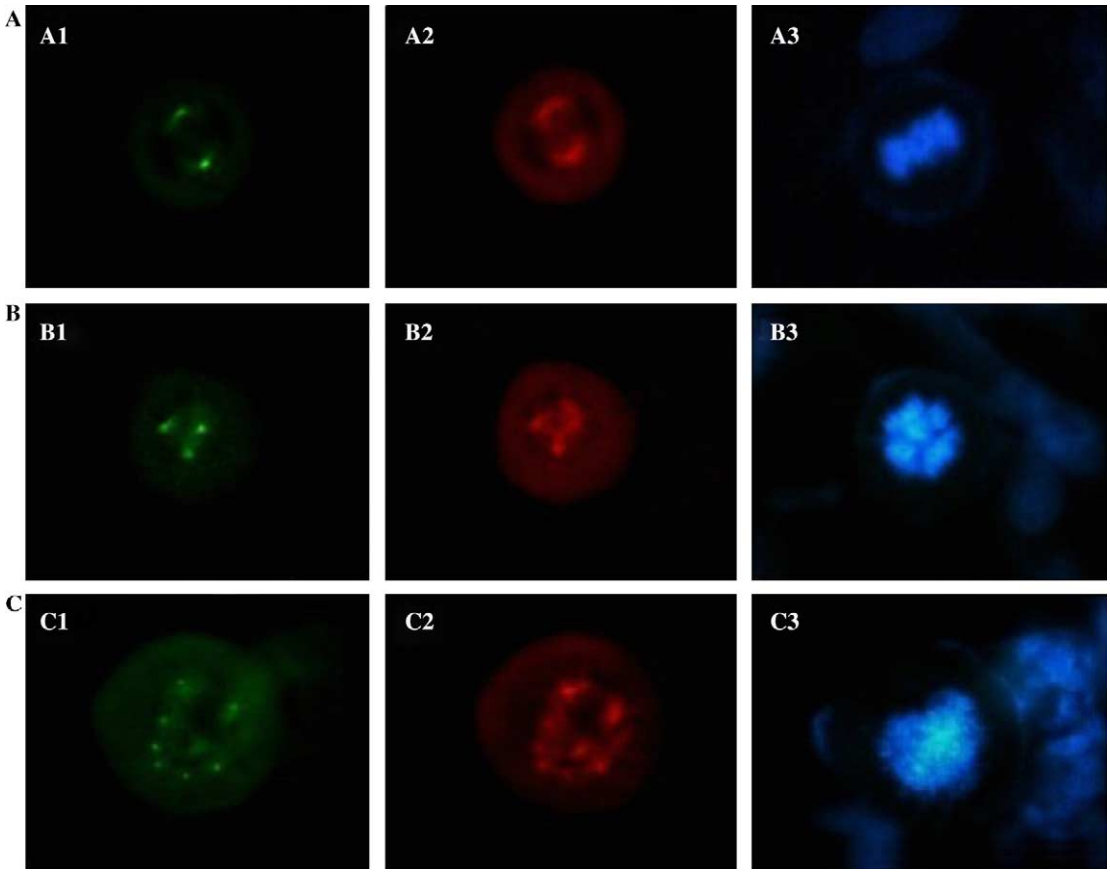

Fig. 6. The phosphorylated TrkA was localized on bipolar and multipolar spindles. (A1, B1, and C1) The spindle poles of mitotic cells were immunostained by monoclonal antibody against γ-tubulin. (A2, B2, and C2) The immunofluorescence staining of phosphorylated TrkA was localized on the bipolar (A2), tripolar (B2), and multipolar (C2) spindles. (A3, B3, and C3) The chromosome was stained by Hoechst 33342.

Discussion

Nerve growth factor may stimulate or inhibit proliferation of neuronal and certain non-neuronal tumors according to the type of tumor. It has been reported that the growth of human glioblastoma multiform (GBM) cell lines can be enhanced by NGF via acting phosphorylated Trk receptor [22,23]. The effect mechanisms of NGF and its high-affinity receptor TrkA in mitogenesis of glioblastoma multiform cell lines have been elucidated: the high-affinity but not low-affinity binding sites mediate signal transduction for clonal growth by means of the tyrosine kinase activity and the following intracellular signaling cascades elicited by the activity. However, whether NGF and its receptor TrkA are directly involved in modulating the mitosis of human tumor cells still remains to be explored. In the present study, we provided the evidence that NGF was colocalized with γ-tubulin at the centrosomes of interphase cells and the spindle poles of mitotic cells. The results demonstrate that NGF is a component of the centrosome and may serve a role in centrosomal functions such as organizing microtubule and generating the spindle pole. Microtubule nucleation is the best-known function of centrosomes. Centrosomal microtubule nucleation is mediated primarily by γ-tubulin ring complexes (γTuRCs) [24,25]. During cell cycle progression, centrosomes "mature" by recruiting additional γTuRCs and several other proteins, resulting in the increase of the nucleation capacity and other functions of the centrosome. Recent work reveals that this organelle is essential for cell-cycle progression and that this requirement is independent of its ability of organizing microtubules [26–28]. Hinchcliffe et al. [29]. have pointed that "core centrosomal structures could bind cell cycle regulatory molecules in a way that activates their function or raises their local concentration to the point that essential reactions occur in a timely fashion." According to this theory, we suggest that NGF binding and concentrating to centrosome could recruit and then activate its receptor TrkA there.

TrkA is the high-affinity receptor for NGF. The high local concentration of NGF facilitates interaction between the ligand and the receptor. NGF can cause phosphorylation of TrkA. The phosphorylated TrkA with the tyrosine kinase activity can phosphorylate the tyrosine of substrate proteins. However, whether TrkA, a receptor protein–tyrosine kinase, could bind to the mitotic spindle and catalyze tubulin tyrosine phosphorylation is unknown. It has been reported that some protein kinases, such as polo-like kinase [30] and Fes tyrosine kinase [31], were required for proper spindle assembly [32,33]. Fes tyrosine kinase is a non-receptor

protein–tyrosine kinase which binds to the mitotic spindle during cell division, interacts directly with both tubulin and MTs, and catalyzes tubulin tyrosine phosphorylation to assemble the mitotic spindle. We have observed that the phosphorylated TrkA was colocalized with α-tubulin at the mitotic spindle. This result demonstrated that the phosphorylated TrkA could play an important role in the mitotic spindle assembly. We have also observed that the localization of phosphorylated TrkA showed dynamic changes. In interphase of U251 cells, the phosphorylated TrkA was localized on the membrane and the processes. While in the mitotic cells, it was colocalized with α-tubulin at the mitotic spindle from prophase to anaphase. From metaphase to late anaphase the staining became weaker. In telophase, as the mitotic spindle disappeared, the weak co-staining was dim on the midbody. The dynamical localization implicated that the TrkA could play different roles under different physiological conditions in the cells. In interphase it may play the role in signal transduction by activating the intracellular signaling cascades. On the other hand, it could play the role in mitosis by promoting mitotic spindle assembly.

In addition to demonstrating the colocalization of NGF and its receptor TrkA with γ-tubulin or α-tubulin, we observed that NGF and TrkA were usually localized at supernumerary centrosomes or multipolar (tripolar, tetrapolar, and so on) spindles in U251 cells. Centrosome defects and genetic instability occur in some low-grade tumors and overexpression of some centrosome-associated proteins, such as Aurora and Rad, induces tumor-like features [34–36]. Centrosome defects may contribute to the cancer development through the generation of chromosome instability [37–40]. This, together with ongoing structural changes in chromosomes, could accelerate accumulation of alleles carrying pro-oncogenic mutations and loss of alleles containing wild-type tumor suppressor genes and thus accelerate the genomic changes characteristic of carcinoma [41]. Whether NGF and TrkA contribute to inducing centrosome abnormalities or/and multipolar spindle formation remains to be elucidated.

In conclusion, our results suggested that NGF and its high-affinity receptor TrkA could play important roles in the growth modulation of glioblastoma multiform cell line U251 cells not only by the classical signal transduction but also by direct involvement in mitosis of the cells.

References

[1] S. Patzke, H. Hauge, M. Sioud, E.F. Finne, E.A. Sivertsen, J. Delabie, T. Stokke, H.C. Aasheim, Identification of a novel centrosome/microtubule-associated coiled-coil protein involved in cell-cycle progression and spindle organization, Oncogene 24 (2005) 1159–1173.

[2] S.W. Krauss, G. Lee, J.A. Chasis, N. Mohandas, R. Heald, Two protein 4.1 domains essential for mitotic spindle and aster microtubule dynamics and organization in vitro, J. Biol. Chem. 279 (2004) 27591–27598.

[3] T. Mayor, Y.D. Stierhof, K. Tanaka, A.M. Fry, E.A. Nigg, The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion, J. Cell Biol. 151 (2000) 837–846.

[4] C.L. Rieder, S. Faruki, A. Khodjakov, The centrosome in vertebrates: more than a microtubule-organizing center, Trends Cell Biol. 11 (2001) 413–419.

[5] W.C. Zimmerman, J. Sillibourne, J. Rosa, S.J. Doxsey, Mitosis-specific anchoring of gamma tubulin complexes by pericentrin controls spindle organization and mitotic entry, Mol. Biol. Cell 15 (2004) 3642–3657.

[6] M. Takahashi, H. Shibata, M. Shimakawa, M. Miyamoto, H. Mukai, Y. Ono, Characterization of a novel giant scaffolding protein, CG-NAP, that anchors multiple signaling enzymes to centrosome and the golgi apparatus, J. Biol. Chem. 274 (1999) 17267–17274.

[7] T. Nishimura, M. Takahashi, H.S. Kim, H. Mukai, Y. Ono, Centrosome-targeting region of CG-NAP causes centrosome amplification by recruiting cyclin E–cdk2 complex, Genes Cells 10 (2005) 75–86.

[8] A. Tritarelli, E. Oricchio, M. Ciciarello, R. Mangiacasale, A. Palena, P. Lavia, S. Soddu, E. Cundari, P53 localization at centrosomes during mitosis and postmitotic checkpoint are ATM-dependent and require serine 15 phosphorylation, Mol. Biol. Cell 15 (2004) 3751–3757.

[9] V.B. Morris, J. Brammall, J. Noble, R. Reddel, P53 localizes to the centrosomes and spindles of mitotic cells in the embryonic chick epiblast, human cell lines, and a human primary culture: an immunofluorescence study, Exp. Cell Res. 256 (2001) 122–130.

[10] V. D'Angiolella, C. Mari, D. Nocera, L. Rametti, D. Grieco, The spindle checkpoint requires cyclin-dependent kinase activity, Genes Dev. 17 (2003) 2520–2525.

[11] C. Vogel, A. Kienitz, I. Hofmann, R. Muller, H. Bastians, Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy, Oncogene 23 (2004) 6845–6853.

[12] D.M. Milne, M. Takahashi, Y. Ono, D.W. Meek, Centrosomal anchoring of the protein kinase CK1delta mediated by attachment to the large, coiled-coil scaffolding protein CG-NAP/AKAP450, J. Mol. Biol. 322 (2002) 785–797.

[13] Y.J Jang, J.H. Ji, J.H. Ahn, K.L. Hoe, M. Won, D.S. Im, S.K. Chae, S. Song, H.S. Yoo, Polo-box motif targets a centrosome regulator, RanGTPase, Biochem. Biophys. Res. Commun. 325 (2004) 257–264.

[14] Z. Dou, X. Ding, A. Zereshki, Y. Zhang, J. Zhang, F. Wang, J. Sun, H. Huang, X. Yao, TTK kinase is essential for the centrosomal localization of TACC2, FEBS Lett. 572 (2004) 51–56.

[15] H.A. Fisk, C.P. Mattison, M. Winey, Human Mps1 protein kinase is required for centrosome duplication and normal mitotic progression, Proc. Natl. Acad. Sci. USA 100 (2003) 14875–14880.

[16] D. Chen, A. Purohit, E. Halilovic, S.J. Doxsey, A.C. Newton, Centrosomal anchoring of protein kinase C betaII by pericentrin controls microtubule organization, spindle function, and cytokinesis, J. Biol. Chem. 279 (2004) 4829–4839.

[17] R.E. Favoni, A. de Cupis, The role of polypeptide growth factors in human carcinomas: new targets for a novel pharmacological approach, Pharmacol. Rev. 52 (2000) 179–206.

[18] M.A. Sortino, F. Condorelli, C. Vancheri, A. Chiarenza, R. Bernardini, U. Consoli, P.L. Canonico, Mitogenic effect of nerve growth factor (NGF) in LNCaP prostate adenocarcinoma cells: role of the high- and low-affinity NGF receptors, Mol. Endocrinol. 14 (2000) 124–136.

[19] S. Descamps, R.A. Toillon, E. Adriaenssens, V. Pawlowski, S.M. Cool, V. Nurcombe, X. Le Bourhis, B. Boilly, J.P. Peyrat, H. Hondermarck, Nerve growth factor stimulates proliferation and survival of human breast cancer cells through two distinct signaling pathways, J. Biol. Chem. 276 (2001) 17864–17870.

[20] L. Dolle, E. Adriaenssens, I. El Yazidi-Belkoura, X. Le Bourhis, V. Nurcombe, H. Hondermarck, Nerve growth factor receptors and signaling in breast cancer, Curr. Cancer Drug Targets 4 (2004) 463–470.

[21] R.J. Bold, S. Rajaraman, J.R. Perez-Polo, C.M. Towsend Jr., J.C. Thompson, Nerve growth factor as a mitogen for a pancreatic carcinoid cell line, J. Neurochem. 64 (1995) 2622–2628.

[22] H.S. Singer, B. Hansen, D. Martinie, C.L. Karp, Mitogenesis in glioblastoma multiforme cell lines: a role for NGF and its TrkA receptors, J. Neurooncol. 45 (1999) 1–8.
[23] E. Oelmann, L. Sreter, I. Schuller, H. Serve, M. Koenigsmann, B. Wiedenmann, D. Oberberg, B. Reufi, E. Thiel, W.E. Berdel, Nerve growth factor stimulates clonal growth of human lung cancer cell lines and a human glioblastoma cell line expressing high-affinity nerve growth factor binding sites involving tyrosine kinase signaling, Cancer Res. 55 (1995) 2212–2219.
[24] E. Schiebel, Gamma-tubulin complexes: binding to the centrosome, regulation and microtubule nucleation, Curr. Opin. Cell Biol. 12 (2000) 113–118.
[25] H.C. Joshi, M.J. Palacios, L. McNamara, D.W. Cleveland, Gamma-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation, Nature 356 (1992) 80–83.
[26] S.P. Blagden, D.M. Glover, Polar expeditions—provisioning the centrosome for mitosis, Nat. Cell Biol. 5 (2003) 505–511.
[27] N.L. Prigozhina, C.E. Oakley, A.M. Lewis, T. Nayak, S.A. Osmani, B.R. Oakley, Gamma-tubulin plays an essential role in the coordination of mitotic events, Mol. Biol. Cell 15 (2004) 1374–1386.
[28] E.H. Hinchcliffe, G. Sluder, "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle, Genes Dev. 15 (2001) 1167–1181.
[29] E.H. Hinchcliffe, F.J. Miller, M. Cham, A. Khodjakov, G. Sluder, Requirement of a centrosomal activity for cell cycle progression through G1 into S phase, Science 291 (2001) 1547–1550.
[30] I. Sumara, J.F. Gimenez-Abian, D. Gerlich, T. Hirota, C. Kraft, C. de la Torre, J. Ellenberg, J.M. Peters, Roles of polo-like kinase 1 in the assembly of functional mitotic spindles, Curr. Biol. 14 (2004) 1712–1722.
[31] C.E. Laurent, F.J. Delfino, H.Y. Cheng, T.E. Smithgall, The human c-Fes tyrosine kinase binds tubulin and microtubules through separate domains and promotes microtubule assembly, Mol. Cell. Biol. 24 (2004) 9351–9358.
[32] D.A. Compton, Spindle assembly in animal cells, Annu. Rev. Biochem. 69 (2000) 95–114.
[33] S. Gadde, R. Heald, Mechanisms and molecules of the mitotic spindle, Curr. Biol. 14 (2004) 797–805.
[34] H. Katayama, W.R. Brinkley, S. Sen, The Aurora kinases: role in cell transformation and tumorigenesis, Cancer Metast. Rev. 22 (2003) 451–464.
[35] D.L. Stenoien, S. Sen, M.A. Mancini, B.R. Brinkley, Dynamic association of a tumor amplified kinase, Aurora-A, with the centrosome and mitotic spindle, Cell Motil. Cytoskel. 55 (2003) 134–146.
[36] M.P. Shekhar, A. Lyakhovich, D.W. Visscher, H. Heng, N. Kondrat, Rad6 overexpression induces multinucleation, centrosome amplification, abnormal mitosis, aneuploidy, and transformation, Cancer Res. 62 (2002) 2115–2124.
[37] C.J. Nelsen, R. Kuriyama, B. Hirsch, V.C. Negron, W.L. Lingle, M.M. Goggin, M.W. Stanley, J.H. Albrecht, Short term cyclin D1 overexpression induces centrosome amplification, mitotic spindle abnormalities, and aneuploidy, J. Biol. Chem. 280 (2005) 768–776.
[38] G.A. Pihan, A. Purohit, J. Wallace, H. Knecht, B. Woda, P. Quesenberry, S.J. Doxsey, Centrosome defects and genetic instability in malignant tumors, Cancer Res. 58 (1998) 3974–3985.
[39] G.A. Pihan, J. Wallace, Y. Zhou, S.J. Doxsey, Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas, Cancer Res. 63 (2003) 1398–1404.
[40] H. Schatten, A.M. Wiedemeier, M. Taylor, D.B. Lubahn, N.M. Greenberg, C. Besch-Williford, C.S. Rosenfeld, J.K. Day, M. Ripple, Centrosome-centriole abnormalities are markers for abnormal cell divisions and cancer in the transgenic adenocarcinoma mouse prostate (TRAMP) model, Biol. Cell 92 (2000) 331–340.
[41] P. Duesberg, R. Li, Multistep carcinogenesis: a chain reaction of aneuploidizations, Cell Cycle 2 (2003) 202–210.