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Short Communication

HIV-1 Nef co-localizes with the astrocyte-specific cytoskeleton protein GFAP in persistently *nef*-expressing human astrocytes

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In T-cells HIV-1 Nef exerts various functions and interacts with actin. In astrocytes interaction of Nef with cellular proteins is poorly understood. Therefore, human astrocytic cell clones stably transfected with *nef*-genes derived from HIV-1 Bru and its myristoylation-defective TH-variant were investigated by confocal laser scanning microscopy for expression of Nef and cytoskeleton proteins actin and GFAP, a marker for activated astrocytes. Myristoylated Nef was detected in cytoplasm, Golgi and plasmamembrane, while non-myristoylated Nef was exclusively cytoplasmic. Nef co-localised with GFAP in the perinuclear region of astrocytes. In contrast, Nef did not interact with actin filaments in human astrocytes. Nef/GFAP interaction could contribute to changes in morphology and activation state of astrocytes shown previously which are both critical for development of astrogliosis in HIV-1 infected brain. *Journal of NeuroVirology* (2001) **7,** 52–55.

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During recent years, evidence arose that the accessory Nef-protein of HIV-1 plays an essential role in the pathogenesis of AIDS and seems to be essential for high viral loads in humans (Deacon *et al*, 1995) and rhesus macaques (Kestler *et al*, 1991). Early after systemic infection, HIV-1 is detectable in the central nervous system (Ho *et al*, 1985). Infection of astrocytes was described by detection of HIV-specific nucleic acids and viral Nef-protein expression (Saito *et al*, 1994) while low production of structural viral proteins was observed (Tornatore *et al*, 1994). Multiply-spliced mRNA transcripts encoding for HIV-1 Tat, Rev and especially Nef were predominantly expressed in astrocytes (Brack-Werner *et al*, 1992; Tornatore *et al*, 1994). In this respect, astrocytes are important target cells for HIV-1, representing a persistent viral reservoir in the brain.

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Functional activities of Nef in different cell types are not yet elucidated and are mostly described in T cells. Cellular localisation of Nef seems to be critical for its function in T cells (Baur *et al*, 1994) and myristoylation is required for its CD4 downmodulating capacity (Harris and Neil, 1994). Co-localisation of Nef with the AP-2 adaptor protein complex suggests a function of Nef in cellular sorting pathways (Greenberg *et al*, 1997). These results elucidate the complexity of functional activities of Nef depending on cell type and intracellular localisation. For this reason we investigated the distribution pattern of Nef in cellular compartments of human astrocytic cells and its interaction with components of the cytoskeleton.

Nef-expressing human astrocytoma cell clones were established by stable transfection of the human astrocytoma cell line U251MG with *nef*-genes derived from the HIV-1 Bru isolate and its myristoylation-defective TH-variant (Kohleisen *et al*, 1999), which was isolated from the permanently HIV-1 infected human astrocytic cell line TH4-7-5 (Brack-Werner *et al*, 1992). Parental U251MG cells and cells containing the *nef*-gene in reverse orienta-

tion (pSGK) were used as controls. Confocal laser scanning microscopy (CLSM, Ziess LSM410, Oberkochen, Germany) was performed on *nef*-expressing and control U251MG clones fixed with 3.5% paraformaldehyde and permeabilised with 0.2% NP40. Nef was detected with monoclonal antibodies (2H12, 2A3, epitope specificity amino acids 171–190). GFAP-specific antibodies were purchased from Sigma (Deisenhofen, Germany) and Cy3- or Cy2-labelled secondary antibodies from Dianova (Hamburg, Germany). Fluorescence channels were acquired sequentially with optimised pinhole at 40 and 60× magnification. Optical section series were acquired in 1-μm steps using identical parameters for *nef*-expressing and control cells. Nuclei were counter-stained with H33258 (Hoechst, Frankfurt, Germany). Actin was detected with FITC-phalloidin (Sigma) using Slow Fade S7461 (Molecular Probes, Eugene, Oregon, USA) to minimise fading. Actin expression was analysed by conventional epifluorescence using a cooled monochrome CCD camera.

For co-immunoprecipitation cell lysates of Bru- and TH-Nef expressing astrocytes and control cells (8×10^6 cells/ml) were incubated with Nef-specific rabbit antiserum (27/89 8′) or pre-immuneserum or a GFAP-specific antibody, respectively and precipitated with Pansorbin (Calbiochem, Frankfurt, Germany). Nef- or GFAP-precipitates were incubated at 95°C in SDS-loading buffer and analysed on a 12% SDS-polyacrylamidegel by Western blot with GFAP- or Nef-specific antibodies, respectively (Kohleisen *et al*, 1992) using ECL system (Amersham-Buchler, Braunschweig, Germany).

High levels of HIV-1 Bru and non-myristoylated TH-Nef were expressed in human U251MG astrocytic cell clones inducing alterations in morphology and growth behaviour of the cells (Kohleisen *et al*, 1999). Single optical sections obtained by CLSM revealed a strong cytoplasmic staining of Bru-Nef concentrated around the nucleus and in the Golgi (Figure 1A,a) of astrocytic cells. Also a distinct Nef-specific staining of the plasmamembrane was detected. In contrast, non-myristoylated TH-Nef was restricted to the perinuclear cytoplasm and was not localised in the plasmamembrane or Golgi (Figure 1A,b). This observation corresponds to the Nef-specific staining pattern observed in permanently HIV-1 infected TH4-7-5 astrocytoma cells, also expressing high-levels of non-myristoylated Nef (Kohleisen *et al*, 1992).

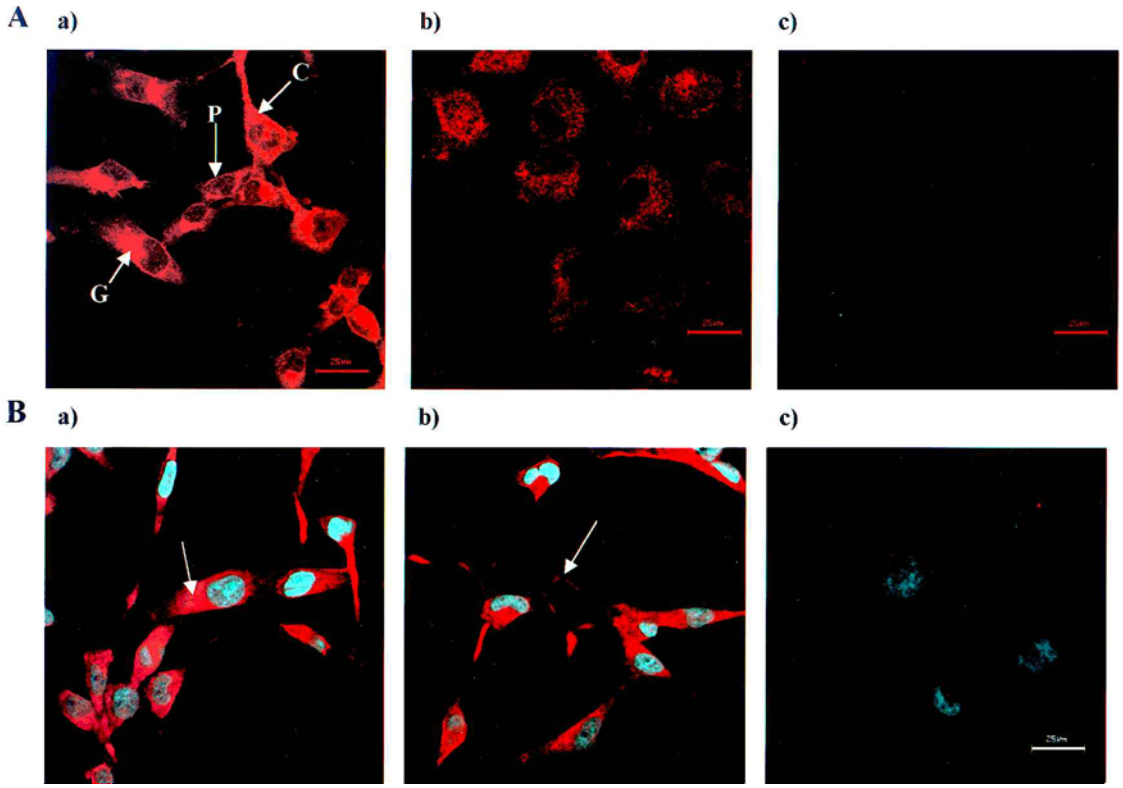

Figure 1 Cellular distribution of Nef (**A**) and GFAP (**B**) in *nef*-expressing U251MG astrocytic cells in CLSM shown as single optical sections (magnification 40×) (**A**) (a) Myristoylated Bru-Nef: localised in cytoplasm (C), Golgi (G), plasmamembrane (P); (b) Non-myristoylated TH-Nef: localised in cytoplasm; (c) Control cells (pSGK). Nuclei are not counterstained. (**B**) GFAP: localised (a) perinuclear (arrow) and (b) in cellular processes (arrow). (c) Control cells (pSGK). Nuclei are counterstained with H33258 (blue).

We could demonstrate the Nef modulates the activation state of astrocytic cells by upregulating the expression of the cytoskeleton protein GFAP which is a marker for activated astrocytes (Kohleisen *et al*, 1999). In CLSM, GFAP was shown to be concentrated around the nucleus and in cellular processes (Figure 1B). Cytoplasmic localisation of GFAP was very similar to the perinuclear Nef-specific staining pattern in stably *nef*-expressing astrocytes. For this reason double labelling experiments were performed in CLSM with Nef- and GFAP-specific antibodies (Figure 2A). Co-localisation of Nef and GFAP was observed in the perinuclear region of astrocytes (yellow stained areas, Figure 2A,c). Similar results were obtained with astrocytes expressing the non-myristoylated TH-Nef (data not shown). Co-immunoprecipitation studies with Nef- and GFAP-specific antibodies revealed weak bands which might be due to an association of both molecules (data not shown).

Actin in *nef*-expressing and control U251MG astrocytic clones appeared as stress fibres and polygonal arrays and also in a non-fibrillary form around the nucleus (Figure 2B,a) as shown by conventional epifluorescence. In contrast to GFAP, Nef is not co-localised with actin (Figure 2B,c), as shown by double labelling experiments.

In fibroblasts and T cells HIV-1 Nef was described to be localised in different cellular compartments like plasmamembrane, cytoplasm or nucleus (Greenberg *et al*, 1997; Kaminchik *et al*, 1994; Yu and Felsted, 1992). In stably *nef*-expressing human astrocytic cells described here myristoylated Nef-protein was detected in the cytoplasm, Golgi and plasmamembrane. In contrast, non-myristoylated TH-Nef was exclusively cytoplasmic, a pattern which was comparable to permanently HIV-1 infected human astrocytoma cells (TH-4-7-5).

Interaction of Nef with cellular proteins like kinases was mainly described in T-cells indicating a role of Nef in modulation of cellular signal transduction pathways (Saksela *et al*, 1995; Sawai *et al*, 1994). In human astrocytic cells we could show a Nef-mediated downregulation of expression level and enzymatic activity of PKC isoforms ε and βII (Ambrosini *et al*, 1999). Complex formation of HIV-1 Nef with actin was observed as myristoylation-dependant process in human B- and T-lymphocytes (Fackler *et al*, 1997; Kaminchik *et al*, 1994), but nothing was described about interaction

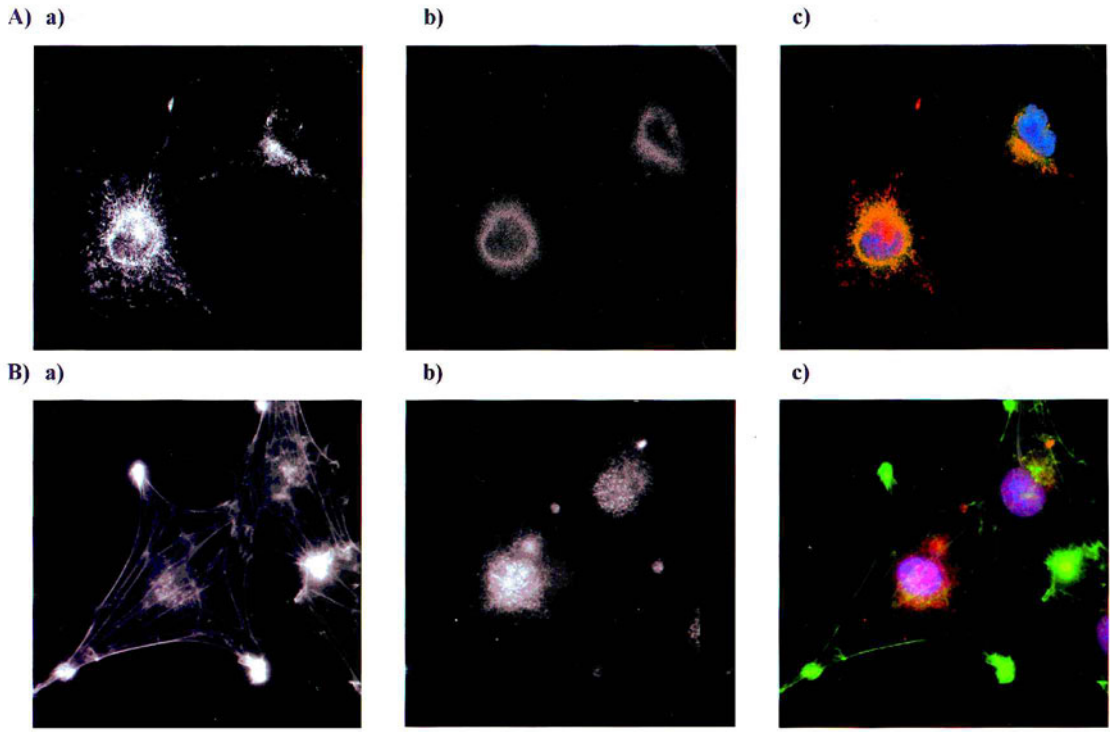

Figure 2 Co-localisation of Nef and GFAP in Bru-*nef* expressing astrocytic cells (magnification 63 ×). (**A**) Double staining with (a) Nef-specific (Cy3, red) and (b) GFAP-specific (Cy2, green) antibodies in CLSM (single optical sections). Perinuclear distribution of Nef and GFAP. (c) Overlay of red and green colour channels: Nef and GFAP are co-localised around the nucleus (area of yellow fluorescence). Nuclei are counterstained with H33258 (blue). (**B**) Actin expression in Bru-nef expressing astrocytic cells analysed by conventional epifluorescence generating a view through the whole cell. Double staining of (a) actin filaments and non-fibrillary actin (FITC, green) and (b) Nef (Cy3, red). (c) Overlay of red and green colour channels: no interaction of Nef and actin. Nuclei are counterstained with H33258 (blue). (a) and (b) are shown in black and white for better demonstration due to higher contrast of the staining pattern.

of Nef with other cytoskeleton proteins like the astrocyte-specific GFAP. Here we could show co-localisation of myristoylated and non-myristoylated HIV-1 Nef with GFAP in the perinuclear region of human astrocytic cells. In contrast, Nef did not co-localise with actin. Cytoskeleton components can influence cell-to-cell adhesion, directional secretion and changes in cell shape (Pearce-Pratt *et al*, 1994). Nef-induced alterations in morphology and growth of stably *nef*-expressing astrocytic cells (Kohleisen *et al*, 1999) suggest that association of Nef and GFAP might influence these effects. Nef-induced upregulation of GFAP expression and interaction of both molecules might contribute to the development of astrogliosis, which is a hallmark of HIV-1 infection in the brain.

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References

Ambrosini E, Slepko B, Kohleisen B, Shumay E, Erfle V, Aloisi F, Levi G (1999). Nef alters the expression of the βII and e isoforms of protein kinase C and the activation of the long terminal repeat promoter in human astrocytoma cells. *Glia* **27:** 143 – 151.

Baur AS, Sawai ET, Dazin P, Fantl WJ, Cheng-Mayer C, Peterlin BM (1994). HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. *Immunity* **1:** 373 – 384.

Brack-Werner R, Kleinschmidt A, Ludvigsen A, Mellert W, Neumann M, Herrmann R, Khim MCL, Burny A, Müller-Lantzsch N, Stavrou E, Erfle V (1992). Infection of human brain cells by HIV-1: restricted virus production in chronically infected human glial cell lines. *AIDS* **6:** 273 – 285.

Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellert A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan JS, Cunningham A, Dwyer D, Dowton D, Mills J (1995). Genomic structure of a attenuated quasi species of HIV-1 from a blood transfusion donor and recipient. *Science* **270:** 988 – 991.

Fackler OT, Kienzle N, Kremmer E, Boese A, Schramm B, Klimkait T, Kucherer C, Mueller-Lantzsch N (1997). Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization. *Eur J Biochem* **247:** 843 – 851.

Greenberg ME, Bronson S, Lock M, Neumann M, Pavlakis GN, Skowronski J (1997). Co-localization of HIV-1 Nef with the AP-2 adaptor complex correlates with Nef-induced CD4 down-regulation. *EMBO J* **16:** 6964 – 6976.

Harris M, Neil JC (1994). Myristoylation-dependent binding of HIV-1 nef to CD4. *J Mol Biol* **241:** 136 – 142.

Ho DD, Rota TR, Schooley RT, Kaplan JC, Allan JD, Groopman JE,. Resnick L, Felsenstein D, Andrews CA, Hirsch MS (1985). Isolation of HTLV-III from cerebrospinal fluid and neural tissue of patients with neurological syndromes related to the acquired immunodeficiency syndrome. *N Engl J Med* **24:** 1493 – 1497.

Kaminchik J, Margalit R, Yaish S, Drummer H, Amit B, Sarver N, Gorecki M, Panet A (1994). Cellular distribution of HIV type 1 protein: identification of domains in Nef required for association with membrane and detergent-insoluble cellular matrix. *AIDS Res Hum Retroviruses* **10:** 1003 – 1010.

Kestler HWI, Ringler DJ, Mori K, Panicali DL, Seghal PK, Daniel MD, Desrosier RC (1991). Importance of the nef gene for maintainance of high virus loads and for development of AIDS. *Cell* **65:** 651 – 662.

Kohleisen B, Neumann M, Herrmann R, Brack-Werner R, Krohn KJE, Ovod V, Ranki A, Erfle V (1992). Cellular localization of nef expressed in persistently HIV-1 infected low producer astrocytes. *AIDS* **6:** 1427 – 1436.

Kohleisen B, Shumay E, Sutter G, Foerster R, Brack-Werner R, Nuesse M, Erfle V (1999). Stable expression of HIV-1 Nef induces changes in growth properties and activation state of human astrocytes. *AIDS* **13:** 2331 – 2341.

Pearce-Pratt R, Malamud D, Phillips DM (1994). Role of cytoskeleton in cell-to-cell transmission of human immunodeficiency virus. *J Virol* **68:** 2898 – 2905.

Saito Y, Sharer LR, Epstein LG, Michaels J, Mintz M, Louder M, Golding K, Cvetkovich TA, Blumberg BM (1994). Overexpression of Nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tissues. *Neurology* **44:** 474 – 481.

Saksela K, Cheng G, Baltimore D (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a a subset of src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J* **14:** 484 – 491.

Sawai ET, Baur A, Struble H, Peterlin BM, Levy JA, Cheng-Mayer C (1994). Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc Natl Acad Sci USA* **91:** 1539 – 1543.

Tornatore C, Meyers K, Atwood W, Conant K, Major E (1994). Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* **68:** 93 – 102.

Yu G, Felsted R (1992). Effect of myristoylation on p27 bef subcellular distribution and suppression of HIV-LTR transcription. *Virology* **187:** 46 – 55.