Virus Research 174 (2013) 8–17

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Cellular localization of BARF1 oncoprotein and its cell stimulating activity in human epithelial cell

Emna Sakka[a], Axel zur Hausen[b], Karim Houali[a,c], Haying Liu[a], Sylvie Fiorini[a], Tadamasa Ooka[a,*]

[a] Laboratoire de Virologie Moléculaire, FRE3011, CNRS, Faculté de Médecine Laennec, Université Claude Bernard Lyon-1, 69372 Lyon, France
[b] Institut für Pathologie, Universitätsklinikum Freiburg, D-79002 Freiburg, Germany
[c] Laboratoire LABAB, Faculté des Sciences Biologiques et Agronomiques, Université Mouloud Mammeri de Tizi-Ouzou, Algeria

ARTICLE INFO

Article history:
Received 13 August 2012
Received in revised form 19 January 2013
Accepted 22 January 2013
Available online 28 February 2013

Keywords:
Epstein–Barr virus
BARF1 protein
Human HaCaT epithelial cell
Nuclear localization
Mitogenic activity

ABSTRACT

BARF1 gene encoded by Epstein–Barr virus is capable of immortalizing the primary monkey epithelial cells and of inducing malignant transformation in human EBV-negative B cell lines as well as rodent fibroblast. This oncoprotein is a secreted protein capable of acting as a powerful mitogen. We have studied the effect of BARF1 protein in transfected or BARF1 protein treated human HaCaT epithelial cells. In BARF1-transfected cells, cell growth was activated and its protein was found both in culture medium and cellular compartment (membrane, cytoplasm and nuclei). When purified BARF1 protein was exogenously added in the cell culture medium of HaCaT cells in absence of fetal calf serum led to its entrance into cells and its intracellular localization in cytoplasm, nuclear periphery and nuclei at 14 h treatment, determined by confocal and immunoelectron microscopy. Cell fractionation confirmed its nuclear localization. Nuclear localization was observed in both systems. More interestingly, purified BARF1 protein p29 exogenously added in the cell culture medium activated cell passage of G1 to S phase. S phase activation by its autocrine activity and its tumorigenic activity would be associated with the development of EBV-associated carcinomas.

1. Introduction

Epstein–Barr virus (EBV) is a ubiquitous herpes virus which represents as a widespread infection in the majority of human populations (Kieff and Rickinson, 2007). EBV is the etiologic infectious agent of mononucleosis (IM) and involved in the pathogenesis of a number of human malignancies of lymphoid and epithelial origin, including Nasopharyngeal Carcinoma (NPC), endemic Burkitt's Lymphoma (BL), Hodgkin's Lymphoma (HD), Non-Hodgkin's Lymphoma (NHL), some T-cell Lymphoma and in lymphoproliferative diseases occurring in immunosuppressed individuals (Kieff and Rickinson, 2007; Young and Rickinson, 2004; Robertson, 2005). There's about 5–10% of gastric carcinoma which is associated with the virus (Takada, 2000).

NPC is tightly associated with EBV (Zur Hausen et al., 1970; Bouzid et al., 1994), almost all NPC contain EBV genome and express several proteins encoded by EBV (Fahraeus et al., 1988; Gilligan et al., 1991; Hitt et al., 1989; Young et al., 1988; Hayes et al., 1999). Among the viral lytic proteins, only BARF1 was consistently expressed this, at high levels in NPC (Decaussin et al., 2000; Seto et al., 2005; Sbih-Lammali et al., 1996). Its expression was detected in EBV-associated GC carcinoma as well as in EBV-immortalized epithelial cells *in vitro* (Danve et al., 2001; Zur Hausen et al., 2000; Chang et al., 2002). BARF1 has a malignant transforming activity in rodent fibroblasts and in human EBV-negative B cells (Wei and Ooka, 1989; Sheng et al., 2001; Ooka, 2005). Its transforming and Bcl2 activating domain was demonstrated between 21st to 56th amino acid sequences by deletion mutants (Sheng et al., 2001). BARF1 has also immortalizing activity on primary primate epithelial cells (Wei et al., 1997). BARF1 has showed a secreted protein in EBV-positive B cells (Fiorini and Ooka, 2008; Cohen and Lekstrom, 1999) as well as BARF1-transfected epithelial and B cells (Cohen and Lekstrom, 1999; Sall et al., 2004). Secreted BARF1 protein (p29) purified from 293 cells infected BARF1 recombinant adenovirus has showed oligomeric hexamer structure determined by recent crystallography study (Tarbouriech et al., 2006) and p29 acts as a powerful mitogen under this form (Sall et al., 2004). Glycosylation and phospholylation is an important step to become biologically functional protein (De Turenne-Tessier and Ooka, 2007; Tarbouriech et al., 2006). This oncoprotein massively secreted in the serum of NPC patients has showed a high mitogenic activity (Houali et al., 2007). Therefore, an important role of BARF1 is suspected in NPC oncogenesis. The p29 protein

* Corresponding author at: Laboratoire de Virologie Moléculaire, FRE 3011, CNRS, Faculté de Médecine Laennec, Université Claude Bernard Lyon-1, Rue Guillaume Paradin, 69372 Lyon Cedex 08, France. Tel.: +33 04 78 77 10 78; fax: +33 04 78 77 10 79.
E-mail address: ooka@sante.univ-lyon1.fr (T. Ooka).

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.01.016

can complex *in vitro* with CSF1 (Colony Stimulating Factor-1) and result in the inhibition of macrophage activation (Strockbine et al., 1998) and can also inhibit the secretion of INF-alpha (Cohen and Lekstrom, 1999). BARF1 is therefore, involved not only in oncogenic mechanism but in immunomodulation as well.

As BARF1 is a secreted protein, its detection in cellular extract becomes difficult. Direct addition of BARF1 in culture medium instead of serum could activate cell cycle by paracrine/autocrine way (Sall et al., 2004). This phenomenon is similar as Tat protein encoded by HIV (human immunodeficient virus), a secreted protein which acts as a growth factor (Ensoli et al., 1990). We are therefore, interested in the modality of BARF1 protein as a factor of growth *in vitro* and *in vivo* on human epithelial cells. We used the human HaCaT epithelial cell line, because its oncogenic activity has never been examined in human epithelial cells so far. In order to understand its localization and its biological properties, we have first transfected BARF1 gene in human epithelial HaCaT cells and secondly, we have treated HaCaT cells directly with purified BARF1 protein. Interestingly, BARF1 was found in nuclear fraction in both systems. BARF1-transfected HaCaT cells showed the activation of cell growth inducing tumor formation in nude mice. Most interestingly, the addition of BARF1 protein in HaCaT cell culture led to activate the cell cycle from passage of G1 to S phase. We discuss the possible role of BARF1 in epithelial oncogenesis in the light of the results obtained in this study.

2. Materials and methods

2.1. Cell culture and antibodies

293-tTA (Tetracycline regulable) and HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FCS (Fetal Calf Serum) and antibiotics as described previously (Sall et al., 2004).

We have used anti-Flag polyclonal antibody and anti-Flag M_2 affinity gel (Sigma, France), which recognizes the Flag epitopes located on Flag-tagged fusion proteins (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C), anti-GFP polyclonal antibodies (Clontech, Germany), anti-BARF1 polyclonal antibody (Decaussin et al., 2000), anti-rabbit or anti-mouse Peroxidase Conjugate IgG antibody and anti-rabbit or anti-mouse FITC antibodies (Santa Cruz, Germany).

2.2. Construction of recombinant vectors

Different primers for constructing recombinant pZip vector (Zhang et al., 1988) and GFP vector which including BARF1, open reading frame and Flag antibody recognized tail was shown in Fig. 1.

Forward primer: 5′cgggatcccagagcaatggccaggttc3′. Reversed primer: (a) BARF-1-Flag-GFP (for C terminal GFP vector): 5′cggggatccgcttgtcatcgtcgtccttgtaatcttgcgacaagtatccagaaac3′. (b) BARF-1-Flag (for pZip vector) 5′cggaattcggatccttacttgtcatcgtcgtccttgtaatcttgcgacaagtatccagaaac3′. The plasmid pC.B-GFP (Fig. 1a) was constructed by inserting the BARF1 coding sequence and GFP coding sequence ligated by Flag peptides, which amplified by PCR with primers BA-1B and BARF1-Flag-GFP, carrying the BamHI restricted site into pfN2-GFP vector. Therefore, it can be recognized by both anti-Flag, anti-BARF1 and anti-GFP antibodies. The plasmid pZip.B-F (Fig. 1b) was generated by inserting the BARF1 coding sequence which was amplified by PCR with primer BA-1B and BARF-1-Flag, carrying adapter for *Bam*HI restriction site into pZip vector. Recombinant proteins were recognized by anti-Flag or anti-BARF1 antibodies.

2.3. Direct and indirect immunoflurescence

HaCaT cells were seeded in six-well plates on cover slips resistant to acetone, and once they reached 60% confluence they were transfected with the three plasmids respectively as above-described by lipofectin reagent transfection kit (GIBCO, BRL).

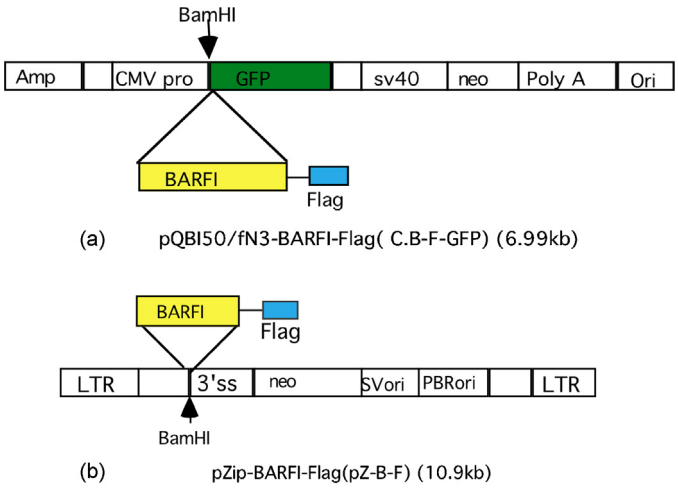

Fig. 1. Cloning of BARF1 cDNA sequence in pZip-Flag vector and pQBI50/fN3-BARF1-Flag (C.B-F-GFP). (a) Fusion protein of BARF1, Flag and GFP was cloned in C-terminal of BARF1 gene. (b) Flag sequence was added in C-terminal of BARF1 in pZip retrovector (48).

(1) For detecting the transient expression, transient transfected cells were seeded in 4-well chamber slides and allowed to adhere. After 24 h, cells were washed twice with phosphate-buffered saline (PBS) and fixed with cold acetone for 15 min. Fixed cells were washed twice with PBS for 5 min.
(2) For detecting the stable expressed cells, cells were transfected and selected by neomycine. Neomycine resistant clones were obtained.

GFP fusion protein was detected directly by UV light microscopy.

2.4. Cell fractionation

1.8×10^6 HaCaT cells were treated by 50 μg of purified p29 protein for 14 h, then cytoplasmic and nuclear fraction were prepared using Nuclear Extraction Kit (Panomics, France). Following the manufacture's instruction, 70 μg of each fraction was loaded onto 12% polyacrylamide gel to analyze the presence of p29. Proteins were transferred onto nitocellulose filter and then the filter was incubated with anti-BARF1 Pep III diluted to 1/1000 with TBS (Decaussin et al., 2000). The proteins were detected by ECL system as previously described (Sall et al., 2004).

2.5. Immunoblotting

Nuclear and cytoplasmic fractions were suspended at 10% w/v in RIPA buffer (0.1% SDS; 0.5% Desoxycholate; 0.5% Triton-X100; 0.4 M NaCl, 5 mM EDTA; 20 mM Tris–HCl, pH 7.6) and sonicated. Fifty or seventy microgram of proteins quantitated by a Bio-Rad protein assay (Bio-Rad Laboratories, Inc.) were diluted with one volume of gel sample buffer (0.2% Bromophenol blue, 4% SDS, 200 mM DTT (dithiothreitol), 20% glycerol, 125 mM-Tris–HCl pH 6.8) and boiled for 5–8 min. Protein samples were separated in 10 or 12% polyacrylamide gels and blotted onto nitrocellulose as previously described (Sheng et al., 2001). Non-specific protein-binding sites were blocked by over-night incubation of blotted filters in TBS buffer with 5% lyophilized BSA (Bovine Serum Albumin, Sigma). The filters were subsequently incubated overnight at 4 °C with rabbit polyclonal anti-BARF1 Pep-III (Decaussin et al., 2000). The filters were then washed and incubated for 1–2 h