Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 346 (2006) 986–991
BBRC
www.elsevier.com/locate/ybbrc

Detection of constitutive heterodimerization of the integrin Mac-1 subunits by fluorescence resonance energy transfer in living cells

Guo Fu [a,b,1], Hua-yan Yang [b,1], Chen Wang [a], Feng Zhang [b], Zhen-dong You [b], Gui-ying Wang [a], Cheng He [b], Yi-zhang Chen [b,*], Zhi-zhan Xu [a,*]

[a] *State Key Laboratory of High Field Laser Physics, Shanghai Institute of Optics and Fine Mechanics, Chinese Academy of Sciences, Shanghai 201800, China*
[b] *Institute of Neuroscience, Department of Neurobiology, Second Military Medical University, Shanghai 200433, China*

Received 31 May 2006
Available online 12 June 2006

Abstract

Macrophage differentiation antigen associated with complement three receptor function (Mac-1) belongs to β_2 subfamily of integrins that mediate important cell–cell and cell–extracellular matrix interactions. Biochemical studies have indicated that Mac-1 is a constitutive heterodimer in vitro. Here, we detected the heterodimerization of Mac-1 subunits in living cells by means of two fluorescence resonance energy transfer (FRET) techniques (fluorescence microscopy and fluorescence spectroscopy) and our results demonstrated that there is constitutive heterodimerization of the Mac-1 subunits and this constitutive heterodimerization of the Mac-1 subunits is cell-type independent. Through FRET imaging, we found that heterodimers of Mac-1 mainly localized in plasma membrane, perinuclear, and Golgi area in living cells. Furthermore, through analysis of the estimated physical distances between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fused to Mac-1 subunits, we suggested that the conformation of Mac-1 subunits is not affected by the fusion of CFP or YFP and inferred that Mac-1 subunits take different conformation when expressed in Chinese hamster ovary (CHO) and human embryonic kidney (HEK) 293T cells, respectively.

Keywords: FRET; Mac-1; Heterodimerization; Spectra; Leukocyte adhesion deficiency (LAD); FR; CFP; YFP; In vivo; CHO

Integrins are a family of cell-surface adhesion molecules that mediate important cell–cell and cell–extracellular matrix interactions. In mammals, 18 α-subunits and 8 β-subunits assemble to form 24 different integrins [1]. Each subunit is composed of a large extracellular domain, a short transmembrane domain, and a short cytoplasmic tail [2,3]. The β_2 subfamily of integrins contains four members that share the common β_2-subunit (CD18) but have distinct α-subunits, α_L (CD11a), α_M (CD11b), α_X (CD11c), and α_D for LFA-1, Mac-1, p150,95, and α_D/β_2, respectively [4,5].

The β_2 integrins are expressed exclusively on leukocytes and mediate important cell adhesion in inflammatory and immune response [6]. The importance of the β_2 integrins is underscored by the disease, leukocyte adhesion deficiency (LAD) type I, a genetic disorder caused by mutations affecting the β_2 chain and resulting in deficiency of β_2 integrins cell surface expression [6]. Association with α-subunit was not detectable in any of the LAD patients who synthesize α- and β-subunits precursor [7].

The α- and β-subunits are biosynthesized as separate α and β intracellular precursors, the precursors associate into αβ complexes, are processed to the mature αβ form, and then transported to the cell surface [8]. Association of the α- and β-chains of β_2 integrins is important for further glycosylation and maturation of the heterodimer and its transport to the cell surface [6]. Furthermore, α_M-subunit can support firm adhesion and spreading of cells but cannot mediate cell migration. In contrast, β_2-subunit cannot mediate firm cell adhesion and spreading but can support

* Corresponding authors. Fax: +86 021 65349829 (Y. Chen).
E-mail addresses: yzchen0928@yahoo.com (Y. Chen), zzxu@mail.shcnc.ac.cn (Z. Xu).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.06.015

migration of cells on recognized ligands [9]. Only the heterodimer of α_M and β_2 subunits exhibits all of the functions responses.

The earliest studies on Mac-1 heterodimerization relied on the photo-affinity labeling, cross-linking studies, Western blot analysis, and immunoprecipitation. Biochemical methods of detection of dimerization, such as immunoprecipitation and cross-linking, require cell disruption and cannot guarantee that dimers are not formed during cell lysis. In contrast, FRET approach allows detection of dimerization in intact living cells [10]. This approach measures the transfer of energy from a donor molecule to an acceptor molecule when they are among 1–10 nm [11]. FRET has been used to show dimerization of LFA-1 subunits [12] and visualize the subcellular compartmentalization of protein–protein interactions in living cells [13,14].

In this study, we proved that there is constitutive heterodimerization of the Mac-1 subunits expressed in living cells and suggested that this constitutive heterodimerization of the Mac-1 subunits is cell-type independent. In addition, we presumed that conformation of the Mac-1 subunits is not affected by the fusion of CFP or YFP and Mac-1 subunits take different conformation when expressed in CHO and HEK293T cells, respectively.

Materials and methods

Plasmids. To create human Mac-1 subunits α_M and β_2 fusion proteins with CFP and YFP, respectively, PCR was used to amplify the α_M and β_2 sequences, which are cloned in the pbluescript3.0 vector. Primers were made to introduce a *Hin*dIII site at the 5′ end (5′-TGGAAGCTTCGA TGGCTCTCAGAGTCCTTC-3′) and to remove the stop codon and added a *Kpn*I site at the 3′ end (5′-TTAGGTACCCGATACTGGG GTTCGGCC-3′) of the α_M. The resulting fragment was cloned into pECFP-N1 vector and pEYFP-N1 vector (Clontech) to obtain the pECFP-N1-α_M and pEYFP-N1-α_M vectors, respectively. The pECFP-N1-β_2 and pEYFP-N1-β_2 vectors were cloned in the same way by using the pECFP-N1 vector and pEYFP-N1 vector. The primers were as follows:(forward, 5′-TGTCTCGAGCCAGCACACCGAGGGACATG-3′; reverse, 5′-AAAGTCGACACATAACTCTCAGCAAACTTGG-3′). All constructs were verified by sequence analysis.

Cell culture and transfection. CHO cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, HEK293T cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were cultured as monolayers in a humidified 5% CO_2 atmosphere at 37 °C. CHO and HEK293T do not express endogenous Mac-1 receptors.

For transient transfection, cells were grown to 50–80% confluence and transfected with appropriate plasmids using LipofectAMINE 2000(Gibco). For immunoblotting experiments, cells were plated into 25 cm^2 tissue culture flasks and used for transient transfection on the second day at about 90% confluence.

Immunoblotting. CHO cells transiently expressing α_M-CFP or β_2-YFP were grown on 25 cm^2 tissue culture flasks to about 90% confluence. CHO cells were washed three times with PBS and were lysed for 30 min on ice, and then the lysates were centrifuged at 12,000 rpm for 10 min. Supernatants were collected, mixed with an equal volume of non-reducing SDS–sample buffer, and subjected to SDS–10% PAGE. Proteins were transferred to nitrocellulose membranes. GFP polyclonal antibody (Santa Cruz) was used to blot CFP and YFP.

FRET measurements with three-channel microscopy. CHO cells or HEK293T cells were plated onto 8-mm^2 glass coverslips in 12-well plates and were transiently transfected with LipofectAMINE2000 (Invitrogen) 24 h later. The cells were washed twice with Hepes-buffered HBSS (pH 7.4) and covered with 0.5 ml HBSS, and then images were taken with a Olympus IX71 inverted microscope equipped with a 60×, NA = 1.45 oil immersion objective lens and cooled charge-coupled device Cascade 512F (roper). Excitation light was delivered by a 75 W Xenon arc lamp. For imaging, the Image-pro®plus version 5.0.1 software (Media Cybernetics) was used. In most of experiments, the excitation intensity was attenuated down to 6% of the maximum power of the light source. Images were acquired using 1×1 binning mode and 100–200 ms integration times.

For quantitative FRET measurements, the method of sensitized FRET was described in detail [15,16]. Images were acquired sequentially through YFP, CFP, and FRET filter channels. Here, the filter sets used were YFP (excitation, 500/25 nm; emission, 545/35 nm), CFP (excitation, 440/21 nm; emission, 480/30 nm), and FRET (excitation, 440/21 nm; emission, 535/26 nm). A dichroic mirror (Olympus) was utilized. The background images were subtracted from the raw images before carrying out FRET calculation. Corrected FRET ($FRET^C$) was calculated on a pixel-by-pixel basis for the entire image by using the following equation: $FRET^C = FRET - (a \times YFP) - (b \times CFP)$, where FRET, CFP, and YFP correspond to background-subtracted images of cells co-expressing CFP and YFP acquired through the FRET, CFP, and YFP channels, respectively [15,16]. The "*a*" and "*b*" are the fractions of bleed-through of YFP and CFP fluorescence through the FRET filter channel, respectively. An alternative quantitative FRET measurements approach as previously described [17] was also used. Briefly, we use the following equation: $FR = [FRET - (b \times CFP)]/(a \times YFP)$ to quantify the FRET signal.

FRET measurements with spectroscopy. Spectroscopic measurements were performed using a spectrograph (Jobin Yvon) and independent optical system developed by ourselves. For excitation, an argon laser (Melles Griot, Carlsbad, CA) was used. CHO cells were plated into 12-well plates 24 h later, cells were transiently transfected with YFP and CFP, CFP–YFP, or α_M-YFP and β_2-CFP. After 24 h, CHO cells were washed with PBS twice, treated with trypsin/EDTA, and suspended with PBS. For each measurement, CHO cells in 800 μl PBS were added to a cuvette. For FRET measurements, emission wavelength scans were performed from 450 to 700 nm with an excitation wavelength of 454 nm. Fluorescence from non-transfected cells and background scattering light brought by the equipment was subtracted from each sample to obtain the specific fluorescence. The emission spectra at 454 nm excitation were normalized to the CFP fluorescence peak at 475 nm [11]. Obtaining the YFP emission spectra due to FRET was performed as previously described [18].

Estimation of physical distance and binding affinity. Details of the model were previously described [17,19]. Briefly, the relationship of the measured FR with the maximal value (FR_{max}) according to the equation $FR = 1 + A_b(FR_{max} - 1)$, where A_b is the fraction of α_M-YFP proteins with an associated β_2-CFP. A_b is given by the 1:1 ligand binding function $A_b = 1/(1 + 2 \times K_d/[D_{free}])$, where K_d is the dissociation constant, $[D_{free}]$ is the concentration of unbound β_2-CFP. FR_{max} and the "effective" dissociation constant $K_{d,EFF}$ were adjusted through minimizing the sum of the squared errors $(FR - FR_{predicted})^2$ across many cells. FRET efficiency was calculated according to equation $E = (FR_{max} - 1)[\varepsilon_{YFP}(440)/\varepsilon_{CFP}(440)]$, and the distances between CFP and YFP were estimated using the equation $R = R_0(E^{-1} - 1)^{1/6}$, with $R_0 = 4.9$ nm.

Statistics. All data were presented as means ± SEM. Data were analyzed using two-tailed Student's *t* test for comparison of independent samples. Differences were considered significant at $p < 0.05$.

Results

Expression of the Mac-1 subunits CFP/YFP fusion proteins

To detect heterodimerization of the Mac-1 subunits in vivo, we constructed cDNA expression vectors for CFP and YFP NH_2-termini fusion proteins of Mac-1 subunits (Fig. 1A). Western blotting demonstrated that both α_M-CFP and β_2-YFP fusion proteins were properly expressed in CHO cells, and no evidence of proteolytic