Short Thesis for the degree of doctor of philosophy (PhD)

Quantitative examination of the hetero- and homodimerization of c-Fos transcription factor in live HeLa cells

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DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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The Examination took place in the Discussion room of the Department of Immunology, Faculty of Medicine, University of Debrecen at 12.00, 11th June, 2014.

Head of the **Defense Committee**: Prof. László Csernoch, PhD, DSc
Reviewers: Prof. Győző Garab, PhD, DSc
László Balkay, PhD
Members of the Defense Committee: Prof. János Matkó, PhD, DSc
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The PhD defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13.30, 15th December, 2015.

1. Introduction

1.1. Members of the Fos and Jun family and their structure

Numerous transcription factors belong to the Fos and Jun family. c-Fos, Fra-1, Fra-2, FosB and its splice variants together with the members of the Jun family (c-Jun, JunB és JunD) form the activator protein-1 (AP-1) group, which bind to the tetradecanoyl-phorbol-acetate (TPA) responsive element on the promoter or enhancer regions of target genes as dimers. c-Jun protein can not only form a heterodimer but also a homodimer. According to a number of *in vitro* studies, the mammalian c-Fos wild type leucine zipper does not promote the formation of homodimers because of the negatively charged residues in the dimerization domain leading to electrostatic repulsion. Isolated c-Fos leucine zippers could form stable dimers at micromolar concentrations; their dissociation constant was 5.6 μM at 25°C. It was demonstrated, that a single amino-acid change in the leucine zipper of c-Fos was sufficient to allow a truncated c-Fos protein to homodimerize and form a complex with DNA.

c-Fos–c-Jun transcription factors consist of four main domains: N-terminal transactivation domain, DNA-binding domain, dimerization domain containing five leucines in the zipper region and the C-terminal transactivation domain. Dimerization and DNA-bindig domains of both families form α-helices, and the basic regions form specific H-bonds and van der Waals interactions with the DNA. Because of the highly conserved DNA-binding and dimerization domains members of the Fos and Jun family have similar DNA-binding and dimerization properties. c-Fos–c-Jun heterodimer binds to a specific heptameric consensus sequence TGA(C/G)TCA with high affinity and with lower affinity to an octameric TGACGTCA sequence i*n vitro*.

1.1. Regulation of the transcriptional activity of Fos and Jun proteins

c-fos and c-jun belongs to early response genes, their transcription is induced shortly after cell stimulus. c-Jun has a low basic expression level. Upon activation, c-Jun positively regulates itself when it binds AP-1 sites within its own promoter. c-Jun can further increase its own induction by dimerizing with another transcription factor, ATF-2. The transcription of c-Fos is regulated by the Elk-1 and cyclic AMP responsive element binding protein /ATF transcription factors. In addition, the binding sites of the serum responsive element (SRE) and the signal transducer and activator of transcription (STAT) can be found in its promoter region as well.

1.2. Activation of c-Fos and c-Jun transcription factors

A number of receptors, cytokines and activators can take part in the activation of c-Fos and c-Jun such as G protein-linked receptors, transforming growth factor β, growth factor receptors or UV radiation and onkoproteins as well. The main pathway of activation of c-Fos and c-Jun is the mitogen activated protein kinase pathway. The activating signal gets to the nucleus, where c-Jun and c-Fos become phosphorylated and activated. In the case of c-Jun activation the activated Jun N-terminal kinase phosphorylates the serins at positions of 63 and 73 in the regulatory region of c-Jun. For the activation of the regulatory region of c-Fos MAP kinase phosphorylates the threonine at position 232.

There are several cis regulatory elements (DNA segments) that mediate c-Fos induction. c-Fos regulator is the Sis-inducible enhancer, which is recognized by STAT3. c-Fos expression can be regulated through thyroid hormone receptor α1 as well, which could induce intestinal tumor. In the case of cell division stimulated by growth factors and cytokines rapid and transient c-Fos transcription can be provoked.

c-Fos expression can be divided into three phases: basic expression, induced expression, which reaches its maximum 15 minutes after TPA treatment, and the decline where transcription level reaches the basic level in two hours.

1.3. Pathological role of Fos and Jun family members

Genes that are up- or downregulated by AP-1 regulate several processes such as differentiation, proliferation, cell survival, hypoxia, angiogenesis and apoptosis. The specific effect of AP-1 depends on which proteins form the dimer, the structure of the promoter region and those transcription factors and activators that bind to the promoter. Overexpression of Fra-1increases the motility and invasion of breast cancer and colon carcinoma cells, but inhibits the tumorigenecity of cervical cancer cells. It was demonstrated that c-Fos, Fra-1 and Fra-2 induces the expression of osteopontin, thrombospondin and CD44, which take part in the metastasis of human breast tumors. In breast carcinoma FosB and Fra-1 binds to tumor supressors. In tumorigenic cells c-Jun dimerizes with c-Fos, but for non-tumorigenic cells it binds to Fra-1. For oral cancer c-Fos forms mainly a heterodimer with JunD. Overexpressed c-Fos holds cyclin D1 in the nucleus thereby preventing its degradation by glycogen synthase kinase 3. Cyclin D1, which is present in the nucleus permanently, induces tumor growth and has a transforming effect. The presence of c-Fos is indispensable for the G_0-G_1 transition. The loss of c-Fos inducibility leads to the inhibition of transcription.

c-Jun has a role in physiological cell growth also: it decreases the expression of the p53 tumor suppressor and that of its substrate, p21, by binding to the promoter region of p53, thereby enhancing cell proliferation. Whereas c-Jun activates transcription of cyclin D1, JunB inhibits it: at the beginning of the G_1 phase phosphorylation of c-Jun and a decline in the level of JunB increase the transcription of cyclin D1. In those fibroblasts where c-Jun was knocked out, cyclin D1 was hardly activated, which led to the termination of the cell cycle because of the improper progression of G_1-S phase transition. Dimerisation tendency of JunB is lower and its DNA-binding activity is weaker than that of c-Jun; therefore, JunB is a less effecient transcriptional activator than c-Jun. An increased level of JunD slows down the proliferation of fibroblasts and holds them in the G_1 phase; therefore, it is considered an inhibitor of cell division.

Numerous studies demonstrated the homodimerization of izolated c-Fos fragments *in vitro* where the protein fragment consisted of the leucine zipper region mainly. The presence of DNA-bound c-Fos homodimers could suggest the possibility that these factors could be transcriptional regulators, and their overexpression might have an important role in cell division and oncogenesis.

2. Aims

c-Jun has the ability to form homodimers with itself and heterodimers for example with c-Fos, but the c-Fos homodimer was found to be unstable. The isolated c-Fos leucine zipper was studied at high concentrations; the truncated protein was able to form stable homodimers *in vitro*. However, none of the studies examined the behavior of the functional protein in live cells. According to the literature c-Fos overexpression could be detected in several tumors, but no data was published on the exact amount of the proteins. Because of the conflicting opinions on the formation of c-Fos homodimers and the high expression level of c-Fos in numerous tumors we were inspired to examine the phenomenon of c-Fos homodimerization.

In our work we asked the following questions:

- Does the c-Fos transcription factor form stable homodimers in live cells?
- How can dissociation constant be determined from FRET data in live cells?
- What are the values of the dissociation constants for the c-Fos homodimer and the c-Fos–c-Jun heterodimer?
- Do c-Fos homo- and heterodimers bind to the chromatin?

3. Materials and Methods

3.1. Cell line, cell culture

Experiments were carried out using HeLa human cervical cancer cells (a kind gift of K. Tóth, German Cancer Research Center). Along with the full length c-Fos proteins a mutant was used where 164 amino acid residues were removed from the C terminal domain (Fos^{215}). As a biological negative control, a deletion mutant of c-Fos lacking the DNA-binding and dimerization domains was used ($c\text{-}Fos^{\Delta\Delta}$); therefore, it is not able to bind to its interaction partners or to the DNA. A construct where donor and acceptor dyes (ECFP-EYFP, or EGFP-mRFP1) were separated by a 7 AA linker was used as positive control for most experiments, which originated high fluorescence resonance energy transfer (FRET) efficiency. For SPIM-FCCS measurements an EGFP-P30-mRFP1 construct was used containing a 30 amino acid long linear proline linker to reduce FRET and to increase the size of the product; addition of the linker increased the diffusion time of the molecule making it more suitable for camera-based SPIM-FCCS measurements (see below).

3.2. Transfection

Confocal microscopic measurements were carried out in 8-well chambered Ibidi coverglasses. For transfection 25 µl of FBS-free RPMI, 2 µl FuGene HD reagent and 0.8 µg DNA were mixed, incubated for 20 minutes at room temperature, then 5 µl of this mix was added to each well. For flow cytometry cells were plated in 24-well cell culture plates; steps of the transfection and the used amounts were the same as for confocal microscopy. For SPIM-FCCS small pieces of cover glass were put into the Petri dishes before seeding the cells. 90 µl FBS-free RPMI was mixed with 0.1 to 6 µg of plasmid DNA and 4 µl FuGene HD reagent and the total amount of the mix was used for transfection in a small Petri dish.

3.3. Calculation of FRET efficiency from confocal images

FRET measurements were performed on a Zeiss LSM 510 confocal microscope. Cell-by-cell ratiometric FRET analysis was performed in Microsoft Excel. For pixel-by-pixel FRET calculations, the RiFRET plugin of the ImageJ software was applied, while FRET histograms and FRET efficiency maps were created in Matlab environment.

For acceptor photobleaching FRET, images of donor and acceptor dye distributions were recorded into two channels. Acceptor dye was then bleached using maximal laser insensity. After photobleaching two-channel image was recorded again. For determination of background intensity a cell-free area of the image was chosen. To reduce noise Gauss-filtering with a 2-pixel radius was used.

3.4. Determination of FRET efficiency using flow cytometry

Flow cytometric measurements were carried out on a FACSAria III instrument. For gating out apoptotic cells and cell debris, forward and side scatter signals were used. For FRET calculations from flow cytometric data, the Reflex software developed at our institute was employed.

3.5. Fluorescence correlation spectroscopy (FCS)

FCS measurements were performed on a modified Olympus FluoView 1000 confocal microscope where the FCS extension equipped with two avalanche photodiodes is attached to the confocal scanning unit. To allow FCS measurements at high EGFP concentrations laser illumination was dimmed by a neutral density filter. Fluorescence autocorrelation functions from the measured fluorescence fluctuations were calculated by an ALV-5000E hardware correlator card. Points for FCS measurements were selected from confocal images; only one point was selected from a cell. From each sample n~30 cells were measured at room temperature, and 6×8 s runs per cell were recorded. Autocorrelation curves were fitted to a two-component

3D diffusion model with triplet correction and EGFP blinking by using the program QuickFit3.0.

3.6. Calibration of fluorescence intensity with FCS

To facilitate the determination of dissociation coefficients (K_d) from FRET data, we developed a method to assess absolute concentrations from fluorescence intensity. In the first step we determined the detection volume of the microscope by using a 130 nM Alexa 488 solution as a standard. From its autocorrelation function the diffusion time and the axial ratio of the ellipsoid-shaped detection volume were determined by fitting. The lateral and axial radii were calculated based on the known diffusion coefficient of the dye; this allowed us to determine the detection volume. From autocorrelation curves of EGFP the particle numbers (N) in the sensitive volume were determined. From these, molar concentrations were calculated. Before every FCS measurement the fluorescence intensity was measured with the imaging detector of the confocal microscope at the site of FCS measurement. Thus, a concentration versus fluorescence intensity calibration curve was generated. The calibration curve was used to assess the concentration of c-Fos-EGFP based on its intensity.

3.7. Transfering the concentration calibration to other instruments

To facilitate comparison of measurements on different days we used fluorescent beads. The fluorescence intensity of 6-µm green calibration beads was used to normalize EGFP fluorescence. The local intensity of the bead at its center corresponded to an EGFP concentration of ~15.4±0.7 µM.

We could also transfer the concentration calibration to flow cytometric measurements. For the calculations we took into account the ratio of the cellular and bead volumes (determined by confocal microscopic 3D sectioning), the different detection efficiencies of the spectra of the bead and EGFP arising from different band pass filters in the flow cytometer and the confocal microscope and the protein fraction localized outside the nucleus. Taking these factors into consideration, the total

intensity of a bead corresponded to a nuclear EGFP concentration of ~1.0±0.1 μM in flow cytometric experiments.

3.8. Determination of the absolute concentration of endogenously expressed c-Fos and c-Jun

For determining the amount of endogeneous and transfected proteins, immunofluorescence labeling was applied. After washing 3× with PBS, cells were fixed with 3.7% formaldehyde (4˚C, 10 minutes), permeabilized with 0.25% Triton and 0.1% TWEEN/TBS (room temperature, 30 min) and blocked with 2% BSA with 0.1% TWEEN/TBS (room temperature, 30 min). Cells were then incubated with mouse anti-c-Fos or mouse anti-c-Jun monoclonal antibody at 20 μg/ml concentration (room temperature, 1 hour), followed by incubation with NL-637-DAMIG polyclonal secondary antibody at 50 μg/ml for 1 hour at room temperature in the dark. Between consecutive steps cells were washed 3× with PBS.

In the case of flow cytometric measurements cells were trypsinized, washed with PBS and from the fixation step the above mentioned protocol was carried on. Flow cytometric measurements were performed on a FACSAria III flow cytometer.

The amount of c-Fos-EGFP was determined by comparing its fluorescence signal to that of the green bead used for concentration calibration. The red signal of the NL637-DAMIG antibody used for immunofluorescence labeling is proportional to the total amount of c-Fos: the endogenous c-Fos in the non-transfected sample, and the endogenous c-Fos + c-Fos-EGFP in the transfected one. Absolute concentration of c-Fos-EGFP was calculated from the green fluorescence signal, which was calibrated with fluorescent beads; then, the absolute concentration of endogenous c-Fos could be defined by comparing the red fluorescence signals.

3.9. Determination of the dissociation constants

Dimerization of donor- and acceptor-labeled proteins results in FRET, based on which the association can be followed. Measurements were carried out on a flow

cytometer; therefore, data from tens of thousands of cells were available for determining the dissociation constant of c-Fos homodimers and c-Fos–c-Jun heterodimers.

In the derivation of dissociation equilibria of the heterodimer we assumed that the heterodimer was much more stable than the c-Fos homodimer; therefore, in the case of c-Fos–c-Jun association we neglected the presence of c-Fos homodimers. The amount of c-Fos homodimers was expressed in terms of the total c-Fos concentration and the dissociation coefficient, then the FRET efficiency was obtained as a function of the total c-Fos concentration and the acceptor-to-donor ratio. For the calculation of the model function we took into account that only those donor molecules give positive FRET that form complex with the acceptor; conversely, donor-donor and donor-endogenous c-Fos pairs contribute zero FRET. For calculating c-Fos–c-Fos equilibria, we have to take into account c-Fos–c-Jun formation as well. Since the heterodimer is more stable, we make the simplifying assumption that all c-Jun molecules present are in complex with c-Fos at the high c-Fos concentrations where c-Fos homodimerization takes place, leaving no free c-Jun. Only donors forming a complex with an acceptor have a positive contribution to FRET.

By plotting the FRET efficiency as a function of the donor-labeled c-Fos concentration FRET titration curves were generated, from which the dissociation coefficient of the c-Fos homodimer and the FRET efficiency for a single donor-acceptor pair was gained.

3.10. Single Plane Illumination Microscopy – fluorescence crosscorrelation spectroscopy

SPIM-FCCS measurements were performed on an in-house built selective plane illumination microscope setup based on the design described in Krieger et al (Optics Express, 2014. 22(3):p2358-2375). 491 nm and 561 nm laser beams were relayed onto a cylindrical lens followed by a projection objective, which formed an approximately 1.3 μm thick light sheet illuminating the cells. Samples were mounted into the sample

chamber hanging from above at an angle slightly below 45° to the light sheet. The fluorescence light was collected with a 60×/NA 1 water dipping objective. The sample fluorescence was split into two color channels using a dichroic mirror and imaged onto an EMCCD-camera which had a pixel size of 400×400 nm² in the object plane. Data acquisition and analysis were performed using our own software QuickFit 3.0. For each measurement, ~100 thousand frames were acquired at a repetition time of ~530 µs. Background acquired without illumination was subtracted from the frames. Photobleaching was corrected on a per-pixel basis by fitting an exponential fall-off; the raw intensity was divided by this fall-off curve, and auto- and crosscorrelation curves of the channel were calculated from it. Intensities were binned for 2×2 pixels to improve the quality of correlation curves. For fitting the data we used a model with two diffusing components for the green and red autocorrelation functions and one component for the crosscorrelation function.

4. Results

4.1. Determination of FRET efficiencies of the dimers

We used full-length c-Jun, c-Fos and its C-terminal truncation mutant Fos^{215} tagged with ECFP or EYFP in confocal microscopic FRET experiments.

4.1.1. FRET analysis with ratiometric method

Images of donor, transfer and acceptor signals were recorded, and FRET efficiencies E between labeled proteins, as well as acceptor-to-donor molecular ratios N_A/N_D were calculated on a pixel-by-pixel or cell-by-cell basis. Cells cotransfected with full length c-Fos-ECFP+c-Fos-EYFP yielded a mean FRET efficiency of E=5.0±0.5%; for c-Fos^{215}-ECFP+c-Fos^{215}-EYFP it was 10.0±0.5%. We also measured FRET efficiency for the c-Fos-ECFP+c-Jun-EYFP and c-Fos^{215}-ECFP+c-Jun-EYFP samples, which was 7.9±0.4% and 15.0±1.1%. The negative control (ECFP coexpressed with EYFP as separate proteins) and the positive control (ECFP-EYFP fusion protein) had mean *E* values of 2.8±0.4% and 48.6±0.8%. These control proteins had a diffuse distribution in the whole cell. The FRET efficiency of the c-Fos–c-Fos (or c-Fos^{215}–c-Fos^{215}) samples was lower than that of the c-Fos–c-Jun or c-Fos^{215}–c-Jun heterodimers, but significantly higher than for the negative control, indicating that c-Fos formed homodimers in these cells.

We determined FRET on a cell-by-cell basis as well, where the mean pixel intensities were determined from the fluorescent region of the cell. Data were grouped into three subsets according to donor concentration based on the fluorescence intensity of the donor: low (<800), medium (800-1200) and high (>1200) expressers. Mean *E* values were plotted as a function of N_A/N_D ratios for the c-Fos^{215}-ECFP+c-Fos^{215}-EYFP and c-Fos-ECFP+c-Fos-EYFP samples. E increases with increasing N_A/N_D ratio because more acceptor-tagged c-Fos molecules are available for donor-tagged ones to form a complex.

4.1.2. By acceptor photobleaching method

The acceptor photobleaching technique is one of the simplest intensity based FRET analysis methods, in which donor intensity of a doubly labeled sample in the presence of the acceptor is compared to the donor signal after photobleaching of the acceptor. The FRET efficiency result (E=48.5%) of the positive control (ECFP-EYFP fusion protein) was used as a standard for ratiometric FRET measurements in the case of the ECFP-EYFP pair. One of the parameters that need to be determined for ratiometric FRET analysis is the so-called α factor, which gives the relative detection efficiency of donor and acceptor signals. Because this factor was difficult to obtain with our experimental setup, the value of this factor was always set to a value so as to get E=48.5% for the positive sample on each measurement day. By using this method the mean FRET efficiency between c-Fos-ECFP + c-Jun-EYFP and c-Fos^{215}-ECFP + c-Jun-EYFP was 8.0±3.6% and 21.2±3.3%, and for negative control it was 1.0±1.5%.

4.2. Determination of absolute concentration from fluorescence intensity

We outlined a method to determine the K_d of interacting proteins in live cells from FRET titration curves. Cytometric FRET efficiencies were plotted as a function of the concentration of dimer forming proteins. This requires calibration of fluorescence intensity as a measure of absolute concentration. Calibration was performed combining confocal microscopy and FCS techniques. Confocal images of cells expressing free EGFP were taken to determine fluorescence intensity in each pixel. Then, FCS measurements were carried out at selected points of the nucleus and from the fitted ACFs the number of particles in the detection volume, N, was determined, from which local EGFP concentrations were calculated. We plotted a calibration line of EGFP concentration vs. fluorescence intensity per pixel, and from the slope of this line the unit of concentration expressed in fluorescence intensity units was gained. Then, the intensity of green fluorescent calibration beads was measured using the same instrument settings, and EGFP intensities were normalized to the intensity of the bead. This way, the molar concentration of EGFP-tagged protein could

be assessed by simply comparing its intensity to that of the bead measured on the same day in the microscopic or flow cytometric setup without having to repeat FCS calibration.

4.3. Formation of c-Fos homodimer and its DNA binding proved by FCS

Diffusing particle concentration vs. fluorescence intensity curves were generated for EGFP and for c-Fos$^{\Delta\Delta}$-EGFP. The similar slopes indicate that equal intensities of EGFP or c-Fos$^{\Delta\Delta}$-EGFP correspond to equal particle number, suggesting that the mutant is monomeric. The full length c-Fos-EGFP protein yielded a slope which was less than half of the monomer values implying the formation of c-Fos homodimers. Particle brightness, *F/N*, is defined as the ratio of the fluorescence intensity (*F*) to the number of particles (*N*). This parameter characterizes the association state of a labeled protein, and it is proportional to the number of fluorophores in a jointly diffusing complex. *F/N* vs. concentration values indicated that c-Fos-EGFP is brighter than EGFP, c-Fos$^{\Delta\Delta}$-EGFP or c-Fos-EGFP+c-Jun-mRFP1, corroborating that c-Fos-EGFP is homodimerized. From the autocorrelation functions (ACFs) molecular diffusion properties were also determined. ACFs were fitted to a model assuming two diffusing components. The average fraction of the slow component for c-Fos$^{\Delta\Delta}$-EGFP, which is not able to bind to the DNA, was lower than that of the heterodimer (c-Fos-EGFP+c-Jun-mRFP1) and for the c-Fos homodimer. This suggests that c-Fos can bind to chromatin not only as a heterodimer, but also as a homodimer.

4.4. Defining the absolute concentrations of endogenous and transfected c-Fos and c-Jun

To measure the total amount of proteins, immunofluorescence labeling was used. Fluorescence intensity of non-transfected cells and cells transfected with c-Fos-EGFP and c-Jun-EGFP was measured by flow cytometry. We detected the green fluorescence signal of c-Fos-EGFP in transfected cells, and compared it to that of the calibration bead to determine the absolute concentration of transfected protein. The

immunofluorescence signal of the non-transfected sample is proportional to the endogenous c-Fos concentration, while that of the transfected one corresponds to the sum of the endogenous and transfected amounts. Thus, using the known concentration of c-Fos-EGFP, we deduced the average concentration of endogenous c-Fos (113±11 nM) and c-Jun (94±10 nM) in HeLa cells.

4.5. Determination of the dissociation coefficient of the c-Fos–c-Jun heterodimer and the c-Fos homodimer

Extent of the dimer formation is proportional to the FRET efficiency; therefore, this parameter was used to detect of association. Flow cytometric FRET experiments were carried out on large cell populations expressing EGFP- and mRFP1-tagged proteins at various concentrations. Cell-by-cell values of FRET efficiency, donor concentration (derived from the FRET-corrected donor intensity by comparison to beads), and acceptor-to-donor molecular ratio were determined. Cells were grouped into classes with approximately constant N_A/N_D ratios and E values of selected N_A/N_D groups were plotted as a function of the donor concentration. Data were fitted to the model function describing the heterodimerization process, taking the presence of endogenous c-Fos and c-Jun into account. The apparent K_d values derived from the fits varied with varying N_A/N_D for c-Fos^{215}-EGFP+c-Jun-mRFP1 and c-Jun-EGFP+c-Fos^{215}-mRFP1 pairs between 10 and 370 nM.

Dissociation coefficients were plotted as a function of the c-Fos–c-Jun ratio for both heterodimer pairs. With an increasing c-Fos:c-Jun ratio the dissociation coefficient decreases, at higher c-Fos:c-Jun ratio where the presence of c-Fos is more significant, we got lower dissociation coefficients for both heterodimers.

In the case of c-Fos homodimerization, curves were fitted taking into account both the homoassociation of c-Fos and its heteroassociation with endogenous c-Jun. The c-Fos–c-Jun complex is more stable than the c-Fos homodimer; therefore, we assumed that all c-Jun molecules were in complex with c-Fos. The fitted model took the donor-donor-, donor-acceptor- and acceptor-acceptor-tagged c-Fos–c-Fos complexes into

consideration; from these only the donor-acceptor-tagged complexes give nonzero FRET. The fit model also takes into account the presence of endogenous c-Fos proteins because these could form complexes with labeled c-Fos and c-Jun proteins as well. In the fits the K_d and E_0 values were linked for data sets with different N_A/N_D values, yielding K_d =6.7±1.7μM and E_0=9.5 ± 0.8% for the homodimerization process. When different N_A/N_D groups were fitted independently, K_d and E_0 ranged between 5.4-9.7 μM and 9.1-11.9%.

4.6. Formation of c-Fos homodimer and its DNA binding proven by SPIM-FCCS

We used fluorescence crosscorrelation spectroscopy (FCCS), the two-color version of FCS to characterize the co-mobility of dimer-forming c-Fos molecules. In FCCS, the auto- and the crosscorrelation functions (CCF) from two molecular species tagged with different colors are determined. Non-zero CCF amplitude indicates that at least a fraction of the labeled molecules are moving together. The ratio of the CCF to ACF amplitudes from a double-labeled sample is proportional to the fraction of molecules forming a complex. FCCS measurements provide 2D interaction and mobility maps.

Measurements were carried out on cells co-transfected with the following protein combinations: c-Fos^{215}-EGFP+c-Fos^{215}-mRFP1, c-Fos^{215}-EGFP+c-Jun-mRFP1, c-Fos$^{\Delta\Delta}$-EGFP+c-Fos^{215}-mRFP1 (negative control) and EGFP-P30-mRFP1. Cells expressing about equal amounts of green and red fluorophores were selected from the concentration range used in FRET experiments (300 nM – 9.6 μM). At each pixel we performed a global FCCS fit to the green and red ACFs and the CCF. The negative control had the lowest (0.06) and the positive control the highest (0.32) apparent dimer fraction, defining the dynamic range of the measurements. The c-Fos–c-Jun heterodimer showed a high apparent dimer fraction (0.22±0.07), whereas the value for the c-Fos^{215} homodimer was slightly smaller (0.16±0.05), but still significantly larger than that of the negative control.

We also analyzed the protein mobility of the green/red dimers that could be extracted from the fits of the CCFs. Using a one component fit the average motility of complexes containing both green and red fluorophores was determined. The diffusion coefficient of the EGFP-P30-mRFP1 fusion protein was ~4.3 μm²/s, whereas for the c-Fos^{215}–c-Fos^{215} homodimer and for the c-Fos–c-Jun heterodimer it was ~0.3 and 0.4 μm²/s, respectively. The presence of a single, slow component for c-Fos homodimers and c-Fos–c-Jun heterodimers indicated that these complexes could bind to slowly moving nuclear components, supposedly the chromatin.

4.7. Analysis of FRET data measured under suboptimal conditions

FRET is a spectroscopic technique often used for investigation of molecular interactions. In ratiometric FRET calculations a pivotal element of the analysis is the determination of the α factor, which is the ratio of the acceptor and donor fluorescence normalized by the number of molecules and by the excitation efficiency. The α factor depends on the spectra of the dyes and the instrument settings. It can be calculated by comparing the fluorescence signal detected in the transfer channel from a given number of acceptor molecules to the signal of an equal number of donor molecules detected in the donor channel. We used donor–acceptor fusion proteins, in which the donor and acceptor dyes are present at a 1:1 ratio. Normalization to the excitation efficiency is usually achieved by applying the same laser line to excite the donor and the acceptor, and division of the resulting fluorescence intensities by the ratio of the extinction coefficients of the two dyes at this wavelength.

For the excitation of ECFP and EYFP 458 and 514 nm laser lines of the confocal microscope were used, which gives an excellent signal-to-noise ratio. In the case of the laser scanning cytometer and the FACSAria flow cytometer the 458 and 514 nm lines were not available. Instead, ECFP was excited at 405 nm, which worked sufficiently, but the excitability of EYFP at this wavelength was low; hence, the exact determination of α factor was not possible. As a FRET standard, the ECFP-EYFP fusion protein was used, which had a FRET efficiency of E=48.5% as determined by

the acceptor photobleaching technique. For the evaluation of laser scanning cytometric and flow cytometric data, the value of α was set to yield 48.5% mean FRET efficiency for the positive control. This α factor value was then also used for analysis of all the other samples. Our measurements were carried out between c-Fos and c-Jun transcription factors. When applying this analysis method, FRET efficiency values measured with different instruments and techniques were in good accordance.

5. Discussion

The activity of AP-1 could be anti-oncogenic, pro-apoptotic, or oncogenic when inducing cell survival. AP-1 dimers, which consist of proteins of Fos, Jun and ATF families, are expressed diversely in different cell types and their regulation is disparate as well. Therefore, every cell type contains a different mixture of AP-1 dimers whose functions could be different as well.

Homodimerization of the isolated c-Fos leucin zipper was reported in several studies *in vitro*. In these studies low stability of the homodimer was reported, and it was assumed that it could not be present in live cells. By combining FRET, FCS and imaging FCCS we demonstrated that c-Fos proteins formed homodimers in live cells, and presented a method for calculating their dissociation constant. The apparent K_d of c-Fos homodimers in HeLa cells was 6.7±1.7 μM. For the c-Fos–c-Jun heterodimer we found a K_d range of 10-370 nM in live cells, which depended on the c-Fos:c-Jun ratio, and on putting the donor and acceptor tags on one or the other protein. The variation of the apparent K_d may be caused by the formation of c-Jun homodimers, which could interfere with the heterodimerization process. At lower c-Fos:c-Jun ratios, where there is excess c-Jun present, the relative amount of c-Jun homodimers is expected to be higher; thus, the amount of available free c-Jun is less and the heterodimerization process could shift toward higher concentrations. At higher c-Fos:c-Jun ratios the c-Jun homodimer is expected to be less abundant. The autofluorescence intensity of HeLa cells in the green channel corresponds to the specific intensity of ~50 nM EGFP, making the K_d for the heterodimers less accurate because the dissociation coefficient for the heterodimer is comparable to this value. Our method to calculate K_d values after concentration calibration by FCS is based on FRET titrations, where the whole procedure was carried out in live cells. In the calculations the concentrations of transfected, fluorescent and endogenous, non-fluorescent proteins were also taken into account. Our concentration calibration method is transferable to measurements performed on different instruments or days by utilizing fluorescent beads as a standard.

A major field of FRET applications is the examination of protein-protein interactions. ECFP and EYFP proteins are a frequently used dye pair in FRET measurements because of their significant spectral overlap. We showed that the fluorescence of ECFP and EYFP excited under suboptimal circumstances could be detected with sufficiently high accuracy and sensitivity by laser scanning and flow cytometry. Thus, by using a standard sample with a known FRET efficiency, the analysis of other FRET samples was possible as well. We compared FRET efficiencies measured by confocal microscopy (optimal ECFP and EYFP excitation), laser scanning cytometry and on flow cytometry (both with suboptimal excitation); FRET results were nearly identical with all the three instruments. We developed a new analysis method where the α factor and the FRET efficiency could be determined simultaneously using a new set of equations. By applying FRET we get information on interactions between proteins but not on the stability of the complex. We used FCS to assess the co-diffusion of molecules, which is a direct indication of stable interaction. Our molecular brightness analysis of FCS data indicated that c-Fos-EGFP, when expressed alone, had twice as high molecular brightness than its dimerization- and DNA-binding-deficient c-Fos$^{\Delta\Delta}$ mutant or the free EGFP dye. When fitted with a slow and a fast diffusion component, the slow fraction of c-Fos was about the same whether expressed alone or together with c-Jun; in contrast, the slow fraction of the c-Fos$^{\Delta\Delta}$ mutant was significantly lower. This hints that the wild type c-Fos binds to the DNA either as a homo- or as a heterodimer.

SPIM-FCCS allowed us to confirm the presence, visualize the distribution and characterize the intranuclear mobility of c-Fos homodimers. Homodimers were stable for at least a few hundred milliseconds. The diffusion coefficient derived from the crosscorrelation curve was ~0.26 $\mu m^2/s$, similar to that of c-Fos–c-Jun heterodimers (0.41 $\mu m^2/s$) and to those data our research group and others observed for other chromatin-binding proteins. The dissociation coefficients for the hetero- and homodimers were lower than that of the EGFP-P30-mRFP1 fusion protein (~4.3 μm2/s) because the latter protein does not bind to the DNA.

The existence of stable c-Fos homodimers capable of chromatin binding brings up the possibility that they may act as transcriptional regulators. The c-Fos homodimer could function as an autonomous transcription factor, or it could also inhibit the DNA binding and function of the c-Fos–c-Jun heterodimer. Thus, the overexpression of c-Fos could have an important role in cell proliferation and in oncogenesis.

6. Summary

The c-Fos and c-Jun transcription factors, members of the activator protein-1 (AP-1) complex form heterodimers, bind to DNA via a basic leucine zipper, and regulate the cell cycle, differentiation, apoptosis, etc. Purified c-Jun leucine zipper fragments could also form stable homodimers, whereas c-Fos leucine zipper homodimers were found to be much less stable in earlier *in vitro* studies. The importance of c-Fos overexpression in tumors and the controversy in the literature concerning c-Fos homodimerization prompted us to investigate c-Fos homodimerization in live cells by fluorescence techniques. We used Förster resonance energy transfer (FRET) and fluorescence (cross)correlation spectroscopy (FCS, FCCS) applied to HeLa cells transfected with fluorescent protein-tagged c-Fos and c-Jun to investigate their homo- and heterodimerization. FRET and molecular brightness analysis by FCS indicated that c-Fos formed homodimers. We developed a method to determine the absolute concentrations of transfected and endogenous c-Fos and c-Jun by combining FCS and immunofluorescence, which allowed us to analyze protein association quantitatively. From FRET data of cells expressing various concentrations of donor- and acceptor-tagged proteins, we determined the apparent dissociation constant of c-Fos homodimers (6.6±1.7 μM) and c-Fos–c-Jun heterodimers (K_d <100 nM) in live cells. Single plane illumination microscopic FCCS confirmed that c-Fos homodimers were stably associated and could bind to the chromatin. Molecular modeling simulations also supported that stable homodimers could form. Our results establish c-Fos homodimers as a novel form of the AP-1 complex, which may be an autonomous transcription factor in c-Fos overexpressing tissues, and could contribute to tumor development.

Registry number: DEENK/211/2015.PL
Subject: Ph.D. List of Publications

Candidate: Nikoletta Szalóki
Neptun ID: R17YB9
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10037769

List of publications related to the dissertation

1. **Szalóki, N.**, Krieger, J.W., Komáromi, I., Tóth, K., Vámosi, G.: Evidence for Homodimerization of the c-Fos Transcription Factor in Live Cells Revealed by Fluorescence Microscopy and Computer Modeling.
   Mol. Cell. Biol. 35 (21), 3785-3798, 2015.
   DOI: http://dx.doi.org/10.1128/MCB.00346-15
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List of other publications

3. Doan-Xuan, Q., **Szalóki, N.**, Tóth, K., Szöllősi, J., Bacsó, Z., Vámosi, G.: FRET Imaging by Laser Scanning Cytometry on Large Populations of Adherent Cells.
Curr. Protoc. Cytom. Suppl. 70., 1-29, 2014.
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IF:4.683

Total IF of journals (all publications): 15,384
Total IF of journals (publications related to the dissertation): 7,843

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

07 October, 2015

DEBRECENI EGYETEM KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA EGYETEMI ÉS NEMZETI KÖNYVTÁR