Technical note

Development of a fast and sensitive method to study transcription factor activation under endogenous conditions in primary mouse T cells applying Alpha technology

Nikolaus Thuille*, Gottfried Baier

Department of Pharmacology & Genetics, Medical University of Innsbruck, Austria

1. Introduction

The regulation of key biological processes in living beings is mainly under the control of a complex system of transcription factors (TFs), proteins that have DNA-binding properties, that can induce or suppress gene transcription (Latchman, 1997; Lee and Young, 2000). To study their interaction with gene promoter regions, the gel-based electrophoretic mobility shift assay (EMSA) is currently the method of choice (Hellman and Fried, 2007; Ramaswami and Hayden, 2015). Time needed to run a gel, imprecise quantification via film blackening and limitation in sample numbers are the disadvantages of this usually radioactivity-based method. To overcome the production of considerable amounts of radioactive waste, chemiluminescence-based systems are offered by different companies (Iwasaki et al., 2008) although in our hands the sensitivity of these systems was not sufficient for studying the DNA-binding capabilities of low-abundance TFs such as NFAT. Our goal was to establish a fast, sensitive, radioactivity-free and good quantifiable assay that can replace the classical EMSA technology. Alpha (Amplified Luminescence Proximity Homogeneous Assay) technology offers the possibility to study the interaction of two molecules bio-conjugated to donor and acceptor beads (Eglen et al., 2008; Vuori et al., 2009). The interaction of the binding partners leads to an approximation of the two beads; upon laser excitation, there is a proximity-dependent chemical energy transfer in a singlet form of oxygen from the donor bead to the acceptor bead. The luminescent signal generated thereby can be easily detected and quantified.

Studying effector functions in primary T cells involves an examination of underlying effector cytokine pathways, such as the Il-2 cytokine. The orchestrated binding of the three TFs NF-kappaB, AP-1 and NFAT to the IL-2 cytokine promotor regulates its expression level and is necessary for the growth, proliferation, and differentiation of naïve T cells into effector T cells (Dienz et al., 2007; Lupino et al., 2012; Liao et al., 2013). In order to test the binding capabilities of NF-kappaB-p50 and AP-1 to DNA-consensus oligonucleotide probes, an adapted EMSA protocol meeting the requirements of the Alpha technology was used. The test conditions were optimized: the amount of oligonucleotide probes and nuclear protein extract concentrations, binding buffers (commercial binding buffers from Active Motif) and binding times were kept equal to the already established EMSA conditions (as described in detail in the M&M section). Instead of P^{32} labeling of the probes, biotinylated DNA sequences were used, which get captured by the streptavidin-coated donor beads. The transcription factor-specific antibodies, which recognize the NF-kappaB and AP-1 proteins in their native forms, were pre-bound to the protein A-coated acceptor beads, leading to a better signal specificity. An overview of the workflow is depicted in Fig. 1.

A stimulation-dependent binding of NF-kappaB and Ap-1 was reproducibly achieved in both primary murine CD3^{+} T cells (Fig. 2A & B) and in the human Jurkat T cell line (Fig. 2C & D), respectively, applying AlphaScreen technology. Murine CD3^{+} T cells treated with a protein kinase C low molecular weight inhibitor (PKC LMWI) have been shown to affect the transactivation of IL-2 promotor- related TFs such as NF-kappaB, AP-1 and NFAT (Evenou et al., 2009). Accordingly, upon stimulation, binding of NF-kappaB to the DNA-oligo was strongly abrogated in inhibitor-treated cells. In order to verify the specificity of the Ap-1 signal in the Jurkat cell line, some of the samples were incubated with mutated oligonucleotide probes, where no or reduced binding should occur. In addition, a set of negative controls were always included to evaluate the background binding: these were samples without oligonucleotide probe, without antibody or without nuclear extract. The results of the AlphaScreen were validated by a parallel EMSA analysis. In order to validate the sensitivity of the assay a data set was included which shows the NF-kappaB DNA binding capabilities in samples of CD3/CD28 stimulated primary murine T cells isolated from wild-type and PKCtheta loss of function (LoF) mutant mice (Thuille, submitted).

Taken together, we have established a fast, nonradioactive, homogeneous immunoassay for quantifying the DNA-binding activities of the TFs NF-kappaB and AP-1 in nuclear extracts of primary murine T cells. The volumes of binding reactions could be adapted to the use of a 384-well microtiter plate format, which is cost effective and enables high throughput screenings. In addition, given the versatility of the Alpha

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* Corresponding author at: Dept. of Pharmacology and Genetics, Medical University Innsbruck, A-6020 Innsbruck, Austria.
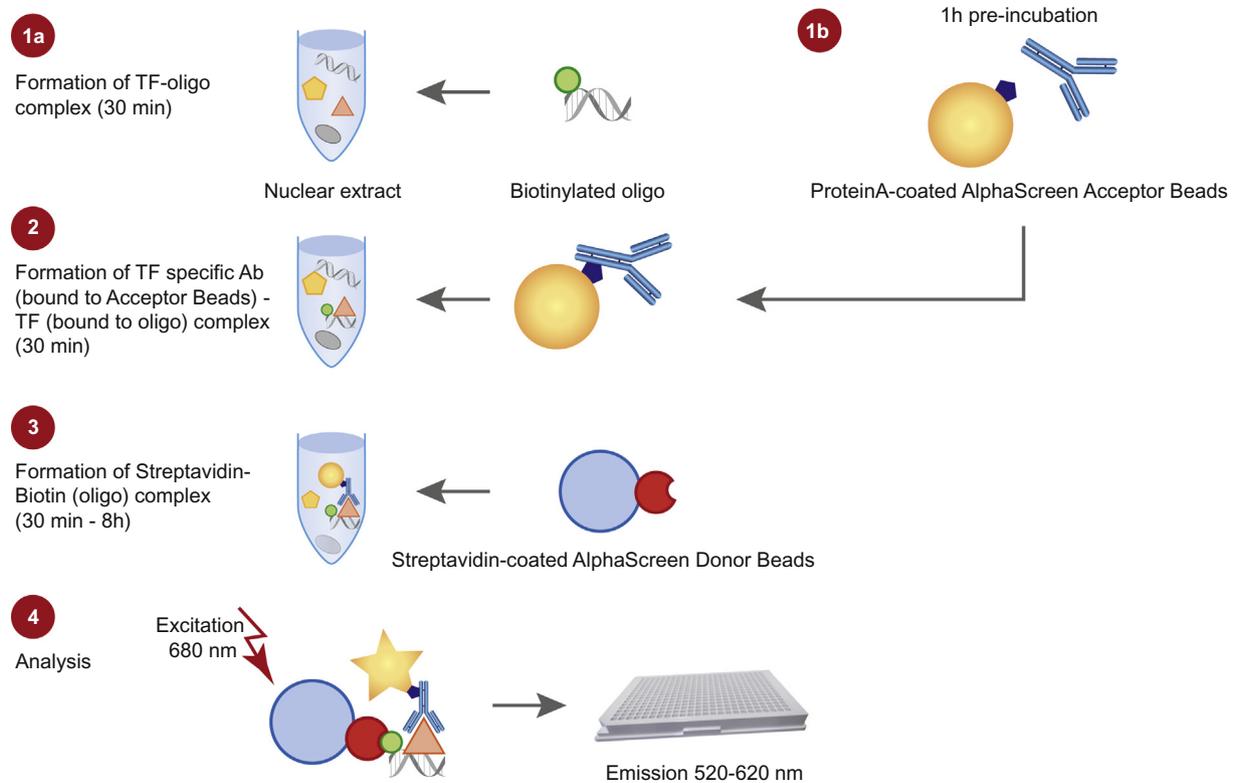*E-mail address:* Nikolaus.Thuille@i-med.ac.at (N. Thuille).

Fig. 1. Workflow of AlphaScreen assay.

technology, detection of DNA-binding TFs can easily be modified by using different antibodies to either full-length or specific epitopes of the protein, or to different tags.

2. Methods

2.1. Reagents

RPMI 1640 medium (w 2.0 g/l NaHCO$_3$, w/o L-glutamine, low endotoxin) was purchased from Biochrom and supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin and 50 U/ml streptomycin. FCS was obtained from Biochrom. The PKC LMWI inhibitor was a gift from Jürgen Wagner, Novartis Pharma, Switzerland.

2.2. Murine CD3$^+$ T-cell isolation with MACS

T cells were isolated from the spleens and lymph nodes with MACS LS columns (Miltenyi Biotec) according to the manufacturer's instructions, using the appropriate protocol for the desired T-cell population: CD3$^+$ T Cell Isolation Kit murine for negative CD3$^+$ T-cell isolation.

2.3. Electrophoretic gel mobility shift assays

Nuclear extracts from unstimulated or stimulated (PDBu/Ionomycin) T cells fractions (2×10^7) were harvested after 16 h as previously described [Pfeifhofer, 2003]. The protein contents of samples were determined with the Bradford method, using the BioRad Protein Assay (BioRad) with BSA (Sigma-Aldrich) as the standard. Nuclear extract proteins (2 μg) were incubated in binding buffers with P^{32}-labeled, double-stranded oligonucleotide probes: NF-kappaB_HIV: 5′-CTGGGGACTTTCCGCT-3′; NF-kappaB_HIV_mut: 5′-CTGTCTACTTT CCGCT-3′; AP-1: 5′-CGCTTGATGACTCAGCCGGAA-3′; AP-1_mut: 5′-CGCT TGATGACTTGGCCGGAA-3′. 3×10^5 c.p.m. of labeled probes were used in each reaction, and band shifts were resolved on 5% polyacrylamide gels. Addition of transcription factor-specific antibodies (super shift) and mutated oligonucleotide probes served as specificity checks.

2.4. AlphaScreen assay

The assay started with a one-hour incubation step of transcription factor-specific antibody (end concentration 20 μg/ml) and protein A-coated acceptor beads (working concentration 50 μg/ml) in Eppendorf tubes on ice. A following washing step of the acceptor beads in PBS removed excess unbound antibodies. In the meantime, frozen samples were thawed and 1–2.5 μg of protein was incubated with 0.5 ng double-stranded biotinylated oligonucleotide probes in binding buffer (containing 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 0.1% BSA, 1 μg poly dI-dC) on ice in Eppendorf tubes for 30 min to enable formation of transcription factor-DNA complexes (24 μl total volume). Thereafter, the protein extract-probe mix was transferred to a 384-well microtiter plate, and 3 μl of acceptor beads were added. The plate was covered and incubated at 4 °C in the dark for 30 min. In the meantime, streptavidin-coated donor beads were prepared (working concentration 50 μg/ml), and 3 μl were added to each well. After a final incubation period of one hour at room temperature in the dark, the plate was read with a PHERAstar FS multiplate reader [BMG Labtech]. A better signal-to-noise ratio could be obtained when the last incubation step was prolonged by 8 h. The final concentration of both beads was 20 μg/ml in a total 30 μl reaction volume.
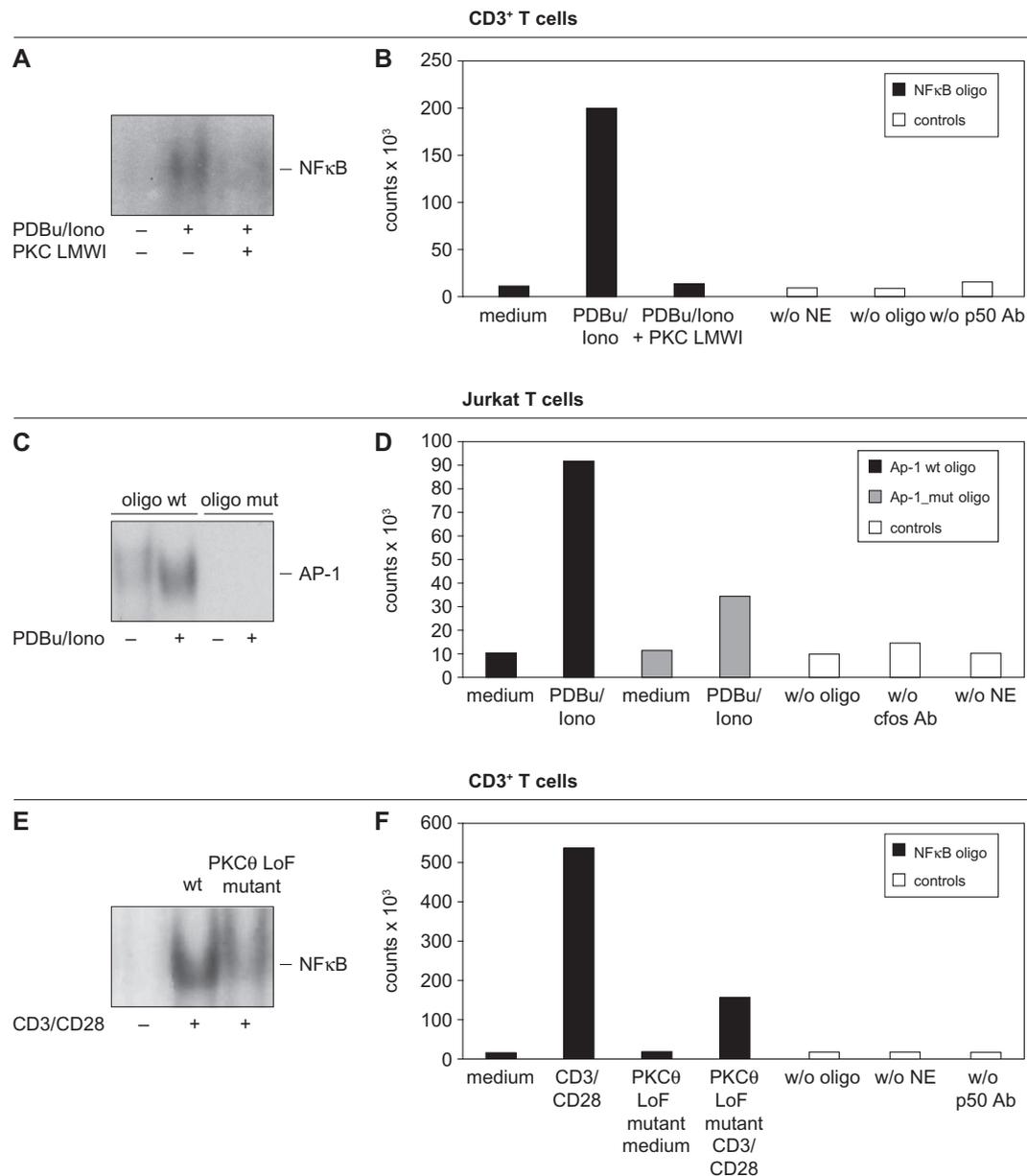
Fig. 2. (A&B) CD3⁺ T cells were isolated and stimulated for 16 h with PDBu/Ionomycin. Half of the stimulated CD3⁺ T cells were treated with PKC LMWI. Nuclear extracts were prepared and analyzed by EMSA (A) and Alpha Technology (B) for NF-κB-p50 binding to DNA. EMSA experiments were repeated at least two times, with similar results. The AlphaScreen experiment shows the mean of three independent experiments +/− SEM. (C&D) Jurkat T cells were stimulated for 16 h with PDBu/Ionomycin. Nuclear extracts were prepared and analyzed by EMSA (C) and Alpha Technology (D) for AP-1 binding to DNA. The specific binding was checked by a mutated DNA-oligo. EMSA experiments were repeated at least two times, with similar results. The AlphaScreen experiment shows the mean of three independent experiments +/− SEM. (E&F) Primary murine T cells isolated from wild-type and PKCtheta loss of function (LoF) mutant mice were stimulated over-night with CD3/CD28 antibodies. NFκB-p50 binding capacities were analyzed by EMSA (E) and Alpha Technology (B) in nuclear extracts. EMSA experiments were repeated at least two times, with similar results. The AlphaScreen experiment shows the mean of three independent experiments +/− SEM.

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