

Calcium dependent activation of the NF-AT transcription factor by p59fyn

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A reporter gene under the control of a T-cell antigen receptor response element was activated in Jurkat cells by antigen receptor triggering or by a combination of phorbol myristate acetate, which activates protein kinase C, and a calcium ionophore. Both these signals were necessary for expression of the reporter gene. When co-transfected with a construct capable of overexpressing the tyrosine kinase p59fyn, the reporter gene was activated by PMA alone. Thus p59fyn could replace the calcium ionophore but not activation of protein kinase C. The activation by p59fyn plus PMA was blocked by EGTA and by the immunosuppressant drug cyclosporin A.

T-cell activation; p59fyn; Tyrosine kinase; Calcium

1. INTRODUCTION

T-cell recognition of specific antigen is achieved by a membrane-associated polymorphic $\alpha\beta$ heterodimer which interacts with peptide antigen complexed with major histocompatibility proteins expressed on the surface of antigen presenting cells. The $\alpha\beta$ heterodimeric recognition molecules have very short cytoplasmic domains and are unlikely to be capable of transducing regulatory signals alone. At least five other transmembrane polypeptide chains are associated with the $\alpha\beta$ heterodimer to form the T-cell antigen receptor complex (TCR). None of the transmembrane polypeptides has any known enzymatic activity (reviewed in [1,2]).

Early events after TCR engagement include protein phosphorylation on tyrosine residues followed by activation of protein kinase C (PKC) and increased intracellular free calcium (reviewed in [2]). At least part of the PKC activation and calcium mobilisation are believed to be mediated by phospholipase C γ which is activated by tyrosine phosphorylation [3]. Phospholipase C γ metabolism of membrane phospholipids results in the release of diacylglycerol, a physiological activator of PKC, and inositol triphosphate which induces a transient release of calcium from intracellular stores (re-

viewed in [4]). Full T-cell activation, however, requires a sustained increase in intracellular free calcium from extracellular sources [5]. Both these signals, PKC activation and calcium mobilisation, are essential and sufficient to activate the antigen response elements of the interleukin-2 promoter which in combination with accessory signals results in IL-2 expression. Expression of IL-2 and IL-2 receptors leads to initiation of an auto-crine growth loop and is a key event in T-cell activation (reviewed in [6]).

The protein tyrosine kinase p59fyn [7,8] is found physically associated with the intracellular domain of the TCR complex and is therefore likely to be involved in TCR signal transduction [9]. Several lines of evidence support this view. Most convincing is that disruption of both homologues of the p59fyn gene in mice results in severe impairment of TCR signaling in thymocytes and to a lesser extent in splenocytes. In particular, thymocytes from p59fyn deficient mice fail to mobilise calcium on TCR engagement [10,11]. In addition, introduction of a constitutively active form of p59fyn into T-cells results in an enhanced proliferative response to TCR triggering [12] and enhanced calcium mobilisation [13].

In the work reported here we have studied the role of p59fyn in IL-2 promoter activation in a Jurkat cell model of T-cell activation. We show that overexpression of p59fyn contributes to activation of a reporter gene under the control of a synthetic promoter containing a multimer of the antigen receptor response element [14] which is recognised by the NF-AT transcription factor. In addition we show that overexpression can, at least in part, replace the requirement for agents which mobilise calcium but not agents which activate PKC.

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Abbreviations: PKC, protein kinase C; TCR, T-cell antigen receptor; CsA, cyclosporin A; CAT, chloramphenicol acetyltransferase; PMA, phorbol myristate acetate; IL-2, interleukin-2.

2. MATERIALS AND METHODS

2.1. Cell culture, transfections and CAT assays

Transfections, activations, protein determinations and CAT assays were carried out as described [15,16]. Each sample resulted from transfection of 1×10^6 cells with $0.5 \mu\text{g}$ NF-AT/CAT and $1 \mu\text{g}$ of either fyn/CMV or vector. To minimize the variability among samples, activations were done on aliquots of a single pool of transfected cells. When p59fyn transfected cells were compared with control cells, the transfection mix, including the indicator plasmids, was prepared, then divided into aliquots to which were added either the p59fyn plasmid or the same vector lacking fyn sequences. Cells were activated 30 h after transfection. EGTA or Cyclosporin A (Sandoz) was added 30 min before activation. OKT3 (ATCC) hybridoma supernatant was enriched in the IgG fraction by affinity purification on protein G-Sepharose (MabTrap, Pharmacia).

2.2. Quantitation of CAT assays

Autoradiograms were scanned using an LKB Ultrascan XL enhanced laser densitometer and evaluated using GSXL software. Each experiment was carried out 3–5 times. The results of representative experiments are shown.

2.3. Plasmids

NF-AT/CAT (a gift of G.R. Crabtree) contains a trimer of the NF-AT binding site of the IL-2 promoter upstream of the CAT gene [17]. Human p59fyn cDNA (a generous gift of M. Toyoshima) was subcloned into the polylinker of the CMV expression vector pcDNA1 (Invitrogen).

3. RESULTS AND DISCUSSION

The T-cell specific transcription factor, NF-AT, is activated by TCR engagement or by a combination of PKC activation and sustained calcium mobilisation. The PKC and calcium mediated signals operate independently on two different subunits of the NF-AT factor. PKC activation results in the induction of expression of the gene coding for one subunit and calcium mobilisation results in translocation of a second subunit from the cytoplasm to the nucleus where the active transcription factor is formed. The activation of both these subunits is essential for NF-AT activity [18].

The reporter plasmid NF-AT/CAT contains the bacterial gene for chloramphenicol acetyl transferase (CAT) under the control of a synthetic promoter consisting of three copies of the NF-AT binding site upstream of a minimal γ -fibrinogen promoter [17]. When introduced into cells of the human lymphoma line, Jurkat, this promoter can be activated by monoclonal antibodies against the TCR/CD3 complex or by nonspecific agonists of the TCR such as phytohemagglutinin. These signals can be mimicked by a combination of phorbol myristate acetate (PMA), which activates PKC, and a calcium ionophore (A23187) [17,19].

Fig. 1 shows the results of CAT assays of extracts of Jurkat cells transfected with the NF-AT/CAT reporter plasmid and treated with various stimuli. In the absence of stimulus, no CAT activity was detected. Treatment with PHA or a combination of PMA and A23187 resulted in significant CAT activity. PMA or A23187 alone had no effect. A combination of anti-CD3 anti-



Fig. 1. Activation of NF-AT in Jurkat cells. CAT assays of protein extracts of Jurkat cells transfected with NF-AT/CAT and activated as shown below each lane in the presence or in the absence of CsA (200 ng/ml) or EGTA (2 mM). 0, non activated; PM, PMA 10 ng/ml; PH, PHA 2 $\mu\text{g}/\text{ml}$; A, A23187 100 ng/ml; 3, OKT3 Mab, 1 $\mu\text{g}/\text{ml}$.

bodies and PMA gave a lower but still significant activation. As has been previously reported [17,20], activation of the NF-AT/CAT construct was completely blocked by removal of calcium ions from the culture medium by EGTA or by the immunosuppressant drug cyclosporin A (CsA), which blocks calcium-dependent pathways of signal transduction.

Fig. 2 shows the results of CAT assays of Jurkat cells co-transfected with the NF-AT/CAT reporter plasmid and a plasmid containing a cDNA encoding p59fyn under the control of a strong constitutively active promoter. In the absence of stimulation or in cells treated with A23187 no CAT activity was detectable. However, after treatment with PMA, significant CAT activity was observed. This activity was approximately 40% of the

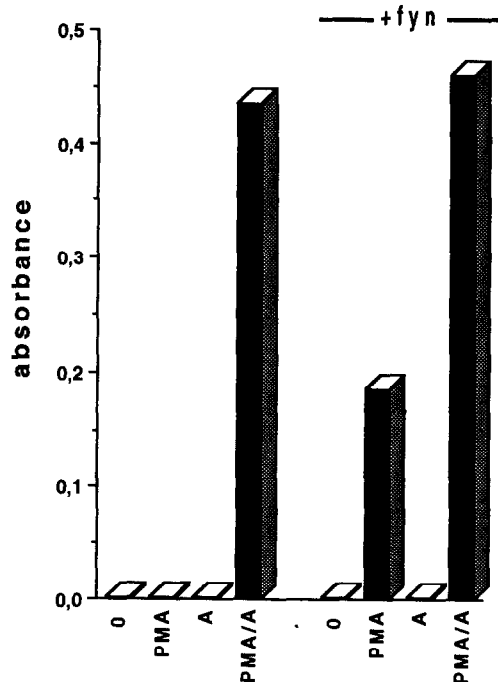


Fig. 2. p59fyn contributes to antigen receptor signaling. Relative absorbance values of acetylated [^{14}C]chloramphenicol in autoradiograms of CAT assays of Jurkat cells co-transfected with NF-AT/CAT and either a control plasmid (left) or an expression plasmid encoding p59fyn (right) and activated as shown below each lane. 0, not activated; PMA, PMA 10 ng/ml; A, A23187, 100 ng/ml. The results are representative of several independent experiments.

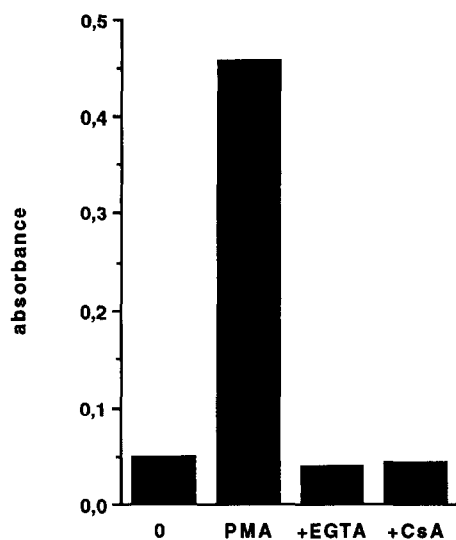


Fig. 3. NF-AT activation by p56fyn is calcium dependent and cyclosporin A sensitive. Relative absorbance values of acetylated [¹⁴C]chloramphenicol in autoradiograms of CAT assays of Jurkat cells co-transfected with NF-AT/CAT and the expression plasmid encoding p56fyn and activated as shown in the absence or in the presence of 2 mM EGTA or 200 ng/ml CsA. PMA, PMA 10 ng/ml.

CAT activity obtained after treatment with PMA and A23187 which fully induce the NF-AT synthetic promoter but was similar to that obtained by anti-CD3 antibodies plus PMA. Thus overexpression of p59fyn, at least partially, replaced the requirement for a calcium mobilising agent but not of PKC activation.

This result supports the hypothesis that p59fyn contributes to TCR signal transduction and that its function is mediated, at least in part, by the transcription factor NF-AT. Furthermore, the fact that it synergises with PMA and not A23187 indicates that it is involved in the calcium-mediated pathway. The signal obtained with PMA treatment of p59fyn transfected cells was never more than 40% of the signal obtained by treatment with PMA plus A23187. This may suggest that p59fyn cannot completely replace the calcium-dependent signal, however in transient cotransfection experiments it is unlikely that all cells which took up the reporter gene also took up the p59fyn expression construct.

To further investigate the role of p59fyn in calcium signaling, Jurkat cells transfected with the NF-AT reporter plasmid and the fyn expressing plasmid were treated with PMA in absence or presence of EGTA to remove calcium from the medium. The results are shown in Fig. 3. EGTA completely blocked the synergism between p59fyn and PMA. EGTA at this concentration has no effect on the activation of calcium independent promoters in these cells [16]. Since p59fyn activation of NF-AT requires the presence of calcium ions in the cell culture medium, it must play a role upstream of the calcium signal. This is in agreement with the fact that thymocytes from p59fyn depleted transgenic mice

do not mobilize calcium in response to TCR engagement [10,11].

Similar results were obtained using the immunosuppressant drug CsA which specifically blocks calcium-dependent signal transduction in T-cells [16]. CsA inhibits the calcium-dependent protease, calcineurin, which is believed to mediate the calcium-dependent activation of IL-2 expression [21]. In the presence of CsA, p59fyn/PMA activation of NF-AT was completely abrogated (Fig. 3).

We conclude that p59fyn mediates activation of IL-2 expression by TCR and that it is involved in TCR modulation of calcium influx from extracellular sources. We have recently shown that a constitutively active form of the CD4-associated kinase p56lck [22] has similar properties [20] and in addition, overexpression of wild-type p56lck also results in calcium mediated activation of NF-AT (Baldari and Telford, unpublished). It is not clear however if p56lck and p59fyn are part of a single signaling pathway or if they function independently.

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