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Calcium-dependent Cyclosporin A-sensitive Activation of the Interleukin-2 Promoter by p56^{lck} *

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T-cell antigen receptor engagement results in suboptimal activation of protein kinase C and a prolonged increase in intracellular free calcium concentration. These signals, in combination with stimulation via accessory molecules usually supplied by the antigen presenting cell, activate expression of interleukin-2 (IL-2) and initiate autocrine growth. The lymphocyte-specific tyrosine kinase p56^{lck} is physically associated with CD4 and is brought into close proximity of the intracellular domain of the antigen receptor by CD4 recognition of the major histocompatibility complex during antigen presentation. p56^{lck} activation enhances and may be essential for antigen receptor signaling. We report that a constitutively active form of p56^{lck} delivers a signal which contributes to IL-2 promoter activation. The signal substituted for a calcium-mobilizing signal in a Jurkat cell model of T-cell activation. The activation was sensitive to EGTA and cyclosporin A, indicating that p56^{lck} functions at an early stage of the calcium-mediated pathway. The transcription factor NF-AT mediated, at least in part, the p56^{lck} activation of IL-2 expression. In addition, activated p56^{lck} synergized with constitutively active p21$^{Ha\text{-}ras}$, which can replace protein kinase C activation, resulting in activation of NF-AT in the absence of external signals.

Activation of resting T-cells is initiated by antigen receptor recognition of specific antigen on the surface of antigen-presenting cells. Early events after antigen receptor engagement include increased protein kinase C activity, increased protein phosphorylation on tyrosine residues and increased intracellular free calcium concentration. These signals, in combination with accessory signals derived from the antigen-presenting cell, result in the activation of expression of interleukin-2 (IL-2)[1] and its receptor leading to autocrine growth and differentiation (reviewed by Clevers *et al.* (1988), Crabtree (1989), and Finkel *et al.* (1991)).

Helper T-cell populations express the CD4 surface marker, which interacts with invariant regions of the class II major histocompatibility complex on antigen-presenting cells. It is believed that this interaction ensures that helper T-cells respond only to antigen expressed in the context of class II MHC (reviewed by Rudd (1990)). The cytoplasmic domain of CD4 is physically associated with the *src*-like tyrosine kinase p56^{lck} (Rudd *et al.*, 1988). During antigen recognition, a complex of TCR, CD4, antigen, and MHC is formed in which p56^{lck} is brought close to the cytoplasmic domain of the TCR.

p56^{lck} is activated by phophorylation of tyrosine at position 394 and down-regulated by phosphorylation of tyrosine at position 505 (reviewed by Sefton (1991)). A mutant form of the protein in which the tyrosine at position 505 has been substituted by phenylalanine, while still functional, can no longer be down-regulated and is constitutively active (Amrein *et al.*, 1988; Marth *et al.*, 1988). Introduction of a construct expressing this mutant into CD4-negative T-helper hybridoma cells results in enhanced response to antigen (Abraham *et al.*, 1991). We report that expression of this mutant in a Jurkat cell model of T-cell activation replaces the requirement for a calcium-dependent signal. In addition we show that activation by p56^{lck} is dependent on extracellular calcium and sensitive to the immunosuppressor cyclosporin A.

MATERIALS AND METHODS

Cell Culture, Transfections, and CAT Assays—Transfections, activations, protein determinations and CAT assays were carried out as described (Macchia *et al.*, 1990; Baldari *et al.*, 1991). For IL-2/CAT experiments each sample resulted from 1×10^7 cells transfected with 6 μg of IL-2/CAT and 4 μg of either F505^{lck}/CMV or the vector lacking the cDNA insert. Jurkat cells do not express detectable IL-1 receptors and do not respond to IL-1. However, these cells can be converted to IL-1 responsiveness by transfection with a construct capable of expressing IL-1 receptors (Baldari *et al.*, 1991; Heguy *et al.*, 1991). In the experiment shown in Fig. 1*A*, 4 μg/sample of such a plasmid construct were included in the transfection. In NF-AT/CAT experiments, each sample resulted from transfection of 1×10^6 cells with 0.8 μg of NF-AT/CAT and 0.6 μg of either F505p56^{lck}/CMV or vector. Transfection efficiencies varied between experiments. For this reason, in each experiment, activations were done on aliquots of a single pool of transfected cells. When p56^{lck}-transfected cells were compared with control cells, the transfection mix, including the indicator plasmid, was prepared, then divided into aliquots to which were added either the p56^{lck} plasmid or the same vector lacking p56^{lck} sequences. Cells were activated 22–30 h after transfection. EGTA or cyclosporin A (Sandoz) was added 30 min before activation.

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 signal obtained due to F505p56^{lck} synergism with either PMA/IL-1 (IL-2 promoter) or PMA (NF-AT promoter) varied in different experiments, presumably due to variation in cotransfection efficiency, but was always between 40 and 100% of the maximal stimulation (PMA/IL-1/A23187 for the IL-2 promoter or PMA/A23187 for the NF-AT promoter). PMA or PMA/IL-1 in the absence of F505p56^{lck} never gave a detectable signal. The results of representative experiments are shown.

Plasmids—IL-2/CAT contains 2000 base pairs of the human IL-2 promoter upstream of the CAT gene (Macchia *et al.*, 1990). 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 (Emmel *et al.*, 1989). F505p56^{lck} (Amrein *et al.*, 1988; Marth *et al.*, 1988) cDNA (a kind gift of R. M. Perlmutter) was subcloned into the *Eco*RI site of the CMV expression vector pcDNAI (Invitrogen). The plasmid T24-ras, encoding p21$^{Ha\text{-}ras}$ with a glycine to valine substitution at

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[1] The abbreviations used are: IL-2, interleukin 2; MHC, major histocompatibility complex; TCR, T-cell receptor; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate.

position 12 and containing the murine leukemia virus long terminal repeat was previously described (Baldari *et al.*, 1992).

RESULTS AND DISCUSSION

IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by the antigen-presenting cell. An IL-2 promoter/chloramphenicol acetyltransferase reporter gene (IL-2/CAT) transfected into the human lymphoma cell line Jurkat can be activated by nonspecific TCR engagement plus a signal supplied by interleukin-1 (IL-1) and its receptor (Baldari *et al.*, 1991; Heguy *et al.*, 1991). The TCR-derived signals could be mimicked by suboptimal concentrations of phorbol myristate acetate (PMA) and the calcium ionophore A23187, which in combination with the IL-1 signal, resulted in maximal CAT expression (Fig. 1*A*) (Baldari *et al.*, 1993). All three signals were required for full CAT activation.

Jurkat cells cotransfected with the IL-2/CAT plasmid and a construct capable of expressing a constitutively active p56^{lck} (Tyr-505 to Phe) mutant protein (Amrein *et al.*, 1988; Marth *et al.*, 1988) responded to a combination of suboptimal PMA plus IL-1 in the absence of the calcium ionophore (Fig. 1*A*). CAT activity was not detectable in PMA/IL-1 treated cells in the absence of mutant p56^{lck}. Thus constitutively active p56^{lck} delivered a signal that contributed to IL-2 promoter activation by replacing the requirement for a calcium-mobilizing signal. Treatment with IL-1 plus ionophore had no effect in the presence or absence of activated p56^{lck}. In the experiment shown in Fig. 1*A*, treatment of control cells with PMA plus ionophore resulted in detectable CAT activity; however, this was not enhanced in the presence of the mutant p56^{lck}. Therefore activated p56^{lck} was unable to replace either the PMA- or IL-1-induced signals.

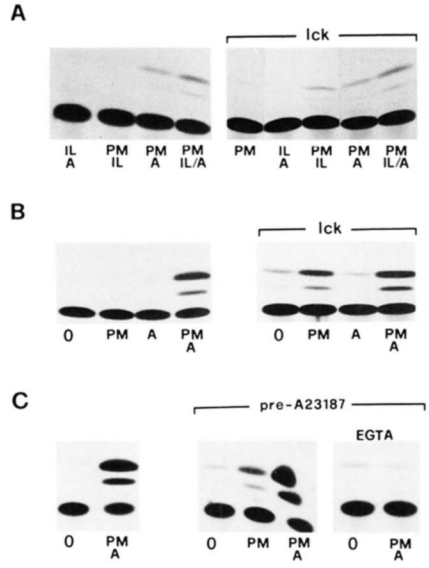

FIG. 1. **p56^{lck} contributes to antigen receptor signaling in IL-2 promoter activation.** *A*, CAT assays of protein extracts of Jurkat cells cotransfected with an IL-2 promoter/CAT construct and either a control plasmid (*left*) or an expression plasmid encoding F505p56^{lck} (*right*) and activated as shown *below* each *lane*. *PM*, 1 ng/ml PMA; *A*, 60 ng/ml A23187; *IL*, 1 ng/ml IL-1β. *B*, CAT assays of Jurkat cells cotransfected with NF-AT/CAT and either a control plasmid (*left*) or an expression plasmid encoding F505p56^{lck} (*right*) and activated as shown *below* each *lane*. *0*, nonactivated; *PM*, 10 ng/ml PMA; *A*, 60 ng/ml A23187. *C*, CAT assays of Jurkat cells transfected with NF-AT/CAT and activated as indicated (*left*) or preactivated for 16 h with the calcium ionophore A23187 (10 ng/ml) then activated as shown *below* each *lane* in the absence (*middle*) or in the presence (*right*) of 3 mM EGTA. *0*, nonactivated; *PM*, 10 ng/ml PMA; *A*, 60 ng/ml A23187. The results are representative of several independent experiments. Quantitation (absorbance units) for *panel A* was as follows: *IL/A* = BD (below detection); *PM/IL* = BD; *PM/A* = 0.25; *PM/A/IL* = 0.61; *lck/PMA* = BD; *lck/IL/A* = BD; *lck/PM/IL* = 0.19; *lck/PM/A* = 0.18; *lck/PM/IL/A* = 0.45. For *panel B* quantitation was: *0* = BD; *PM* = BD; *A* = BD; *PM/A* = 2.78; *lck/0* = 0.33; *lck/PM* = 1.55; *lck/A* = 0.22; *lck/PM/A* = 2.58. For *panel C* quantitation was: *0* = BD; *PM/A* = 4.90; *Pre-A/0* = 0.20; *Pre-A/PM* = 1.26; *Pre-A/PM/A* = 6.24; *Pre-A/0/EGTA* = 0.19; *Pre-A/PM/A/EGTA* = 0.14.

A trace of NF-AT/CAT activity was detected in p56^{lck}-transfected cells in the absence of costimulus, whereas no CAT activity was detected in control cells treated for 9 h with A23187 (Fig. 1*B*). This might suggest that p56^{lck} does more than just substitute for calcium. A more likely explanation, however, is that the p56^{lck} produced during the recovery period after transfection is capable of synergism with the low level of protein kinase C activity present in untreated cells. In the experiments shown in Fig. 1 (*A* and *B*), the cells were transfected, then allowed to recover for 22 h before activating with the various stimuli for 9 h. Since the *lck* coding sequences are under the control of a constitutive promoter, p56^{lck} is likely to be produced during most of the recovery period. In the experiment shown in Fig. 1*C*, we have assayed the effect of prolonged treatment with a low concentration of A23187 on NF-AT/CAT-transfected cells. A23187 was added to the cells 6 h after transfection, and the cells were harvested after an additional 24-h incubation. A low level of CAT activity, similar to that obtained from untreated *lck*-transfected cells, was detected. EGTA added after 16 h of A23187 treatment had no effect on this low signal, suggesting that it reflects CAT protein produced during the recovery period.

Further confirmation of a role for p56^{lck} in calcium-mediated signaling comes from the experiments shown in Fig. 2. Removal of calcium from the culture medium by chelation with EGTA before activation completely blocked NF-AT/CAT activation by PMA in p56^{lck}-transfected cells (Fig. 2*a*). The trace amount of activity detected in the absence of external stimuli was, however, unaffected, supporting the hypothesis that this activity is due to CAT protein produced during the recovery period before the addition of EGTA. The activity of NF-AT in T-cells is extremely sensitive to the immunosuppressor cyclosporin A, which blocks specifically calcium-dependent transcriptional regulation (Baldari *et al.*, 1991; Mattila *et al.*, 1991). At a concentration known to block NF-AT activation, cyclosporin A blocked completely the synergism between p56^{lck} and PMA (Fig. 2*b*). The activity detected in the absence of external stimuli was again found to be resistant, indicating synthesis of CAT protein before inhibition of NF-AT. Neither removal of calcium with EGTA

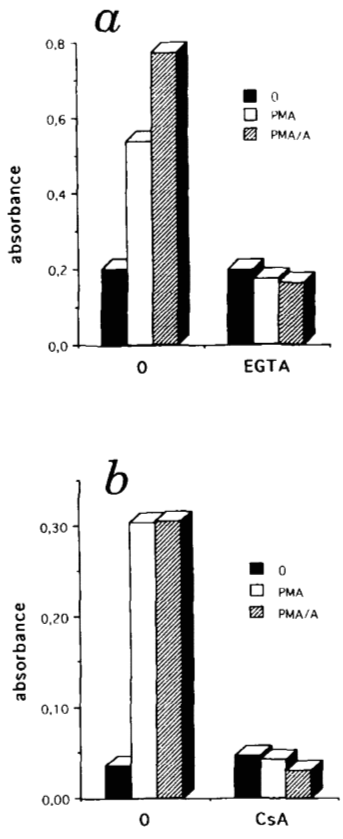

FIG. 2. **NF-AT activation by p56[lck] is calcium-dependent and cyclosporin A sensitive.** *a*, relative absorbance values of acetylated [^{14}C]chloramphenicol in autoradiograms of CAT assays of Jurkat cells cotransfected with NF-AT/CAT and the expression plasmid encoding F505p56[lck] and activated as shown in the absence or in the presence of 3 mM EGTA. *b*, relative absorbance values of acetylated [^{14}C]chloramphenicol in autoradiograms of CAT assays of Jurkat cells cotransfected with NF-AT/CAT and the expression plasmid encoding F505p56[lck] and activated as shown in the absence or in the presence of 500 ng/ml cyclosporin A. *PMA*, 10 ng/ml PMA; *A*, 60 ng/ml A23187.

nor treatment with cyclosporin A had any effect on PMA activation of an SV40 early promoter-CAT construct (Baldari *et al.*, 1991), excluding general toxic effects of these substances.

The data presented thus far show that expression of constitutively active p56[lck] results in a pattern of promoter activation indistinguishable from that of the calcium ionophore A23187. In addition, its sensitivity to EGTA and cyclosporin A indicate a functional role for this kinase at an early stage of the calcium-dependent activation. This is consistent with a recent report that transfection of a p56[lck] plasmid restores TCR signaling and calcium flux in a p56[lck] defective T-cell line (Strauss and Weiss, 1992). An oncogenic form of the prototype of this family of tyrosine kinases, v-*src*, has been shown to modulate intracellular free calcium (Niklinska *et al.* 1992) levels and to induce IL-2 expression in T-cells (O'Shea *et al.* 1991). However, very little p60[src] is expressed in T-cells and there is no evidence of p60[src] involvement in TCR signal transduction. An analogy may be drawn with the epidermal growth factor receptor tyrosine kinase, which induces LTB4-mediated opening of cell membrane calcium channels (Peppelenbosch *et al.*, 1992). p56[lck] activity was, however, unaffected by nordihydroguaiaretic acid, which blocks the production of LTB4 by inhibiting 5-lipoxygenase (data not shown).

The small GTP-binding proteins of the *ras* family have been shown to play a role in the protein kinase C pathway of TCR signaling (Downward *et al.*, 1990; Baldari *et al.*, 1992). T24, a constitutively active Val-12 mutant of p21[Ha-ras] (Santos *et al.*, 1982) can specifically replace PMA treatment in activation of both the IL-2 promoter (Baldari *et al.*, 1992; Rayter *et al.*, 1992) and the NF-AT transcription factor (Baldari *et al.*, 1993). Since p21[Ha-ras] and p56[lck] appear to have complementary activities in T-cells, we reasoned that expression of constitutively active forms of both proteins should result in NF-AT activation in the absence of external signals. Fig. 3 shows the results of cotransfection of NF-AT/CAT with a plasmid capable of expressing the T24 oncogenic form of p21[Ha-ras] (Baldari *et al.*, 1992), the p56[lck] expression plasmid, or both constructs together. As predicted, p56[lck] acted synergistically with T24-ras to activate the NF-AT promoter. The CAT activity observed was less than that obtained after stimulation with PMA and A23187, as might be expected since in cotransfection experiments not all cells that received the reporter gene were likely to have received both the T24 and p56[lck] expression plasmids. It cannot be excluded, however, that p56[lck] and p21[Ha-ras] do not replace completely the PMA and ionophore signals.

The role of p56[lck] in antigen stimulation of T-cells is controversial. It has been variously reported to deliver a negative down-regulating signal (Newell *et al.*, 1990), to be essential for TCR signaling (Gleichenhaus *et al.*, 1991) and to enhance signals delivered by other TCR components (Veillette *et al.*, 1990; Abraham *et al.*, 1991). Recent data suggest a model that may reconcile previous data (Haughn *et al.*, 1992). In this model, CD4 sequesters p56[lck] making it unavailable for TCR signaling in the absence of MHC. Thus cross-linking of CD4 prior to antigen presentation results in lowered TCR signaling. In addition, anti-TCR-CD3 antibodies are less effective in CD4 expressing cells than in CD4$^-$ cells. However, when antigen is presented in the context of MHC, CD4 recognition of MHC brings p56[lck] into close proximity of the TCR$\alpha\beta$·CD3 complex, where it can be activated and deliver a signal. The model implies a prerequisite role for p56[lck] in TCR

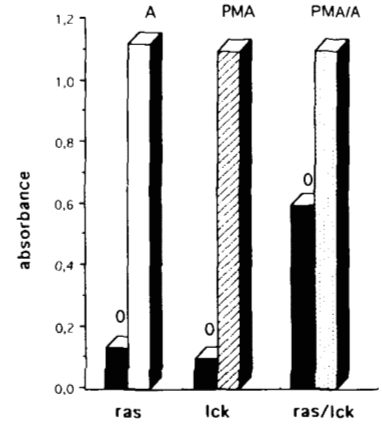

FIG. 3. **p21[Ha-ras] and p56[lck] synergism in the activation of NF-AT.** *Relative absorbance* values of acetylated [^{14}C]chloramphenicol in autoradiograms of CAT assays of Jurkat cells cotransfected with NF-AT/CAT and either an expression plasmid encoding constitutively active p21[Ha-ras] or the expression plasmid encoding F505p56[lck] or both expression plasmids and activated as shown *above* each column. *0*, nonactivated; *PMA*, 10 ng/ml PMA; *A*, 60 ng/ml A23187. NF-AT was used at 0.5 μg/10^6 cells, T24-ras at 0.8 μg/10^6 cells, and F505p56[lck] at 0.3 μg/10^6 cells. The total DNA was adjusted to 1.6 μg/10^6 cells using the appropriate vector (pcDNAI with T24-ras and DOL$^-$ (Korman *et al.*, 1987) with p56[lck]). A time course was carried out, with activations at 8, 22, or 30 h after transfection. Although the results were similar at all times tested, the Ras activation and Ras synergism with p56[lck] were highest at 30 h. The result presented shows the 30-h time point.

signaling. Our data are consistent with this model and show that activated p56^{lck} can deliver a signal contributing to IL-2 activation. In addition the data indicate that p56^{lck} is involved in TCR-mediated triggering of increased intracellular free calcium.

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