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CUTTING EDGE

Cutting Edge: A Chemical Genetic System for the Analysis of Kinases Regulating T Cell Development[1]

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***To understand the regulatory activities of kinases in vivo requires their study across a biologically relevant window of activity. To this end, ATP analog-sensitive kinase alleles (ASKAs) specifically sensitive to a competitive inhibitor have been developed. This article tests whether ASKA technology can be applied to complex immunological systems, such as lymphoid development. The results show that when applied to reaggregate thymic organ culture, novel $p56^{Lck}$ ASKAs readily expose a dose-dependent correlation of thymocyte development with a range of $p56^{Lck}$ activity. By regulating kinase activity, rather than amounts of RNA or protein, ASKA technology offers a general means for assessing the quantitative contributions to immunology of numerous kinases emerging from genomics analyses. It can obviate the generation of multiple lines of mice expressing different levels of kinase transgenes and should permit specific biological effects to be associated with defined biochemical activities. The Journal of Immunology,* 2003, 171: 519–523.**

The pivotal role of kinases in biology is clear (1, 2). Nonetheless, we are often ignorant of their biologically relevant substrates and the quantitative window of kinase activity required to phosphorylate them. It is seldom clear when biological processes have graded responses to kinase dosage or "all-or-nothing" responses to thresholds of kinase activity, and thus it is difficult to predict the effect of modest differences in kinase activity that will characterize human genetic variation.

An improved understanding ideally requires a capacity to regulate specific kinase activities within complex mammalian systems. In this regard, a recent technological innovation described ATP analog-sensitive kinase alleles (ASKAs)[4] that can accommodate ATP and function normally, but that additionally have high affinity for large ATP analogs, such as 4-amino-1-*tert*-butyl-3-(1′-naphthyl)pyrazolo[3,4-*d*]pyrimidine (NaPP1), that compete for entry into the ATP binding site (3, 4). By contrast, the active site of natural kinases is too small to accommodate such inhibitors (3) (Fig. 1*a*). Thus, ASKA technology theoretically permits one to regulate the biological effects of any chosen kinase.

This notwithstanding, the application of ASKA technology to complex mammalian biology faces potentially serious uncertainties. These include the expression levels of ASKAs achievable in primary cells and whether the doses of inhibitor needed to gain access to relevant cells within a multicellular organ cause nonspecific biological dysregulation: in sum, will the achievable expression levels of the ASKAs and the practical concentrations of inhibitor combine to regulate kinase activity across a biologically relevant range? To resolve these issues, this article examines the practicality of ASKA technology for studying T cell development in reaggregate fetal thymic organ culture (RTOC).

Stages of thymocyte development are readily marked by CD4 and CD8 expression (5). Early $CD4^-CD8^-$ "double-negative" (DN) thymocytes mature into $CD8^{low}$ cells (immature single-positive cells (ISPs)) that become CD4CD8 double-positive (DP) (6), and finally $CD4^+$ or $CD8^+$ single-positive (SP) cells. The DN-ISP-DP transition depends on the TCR β-chain contributing to a pre-TCR (7–9), while the DP-SP transition depends on TCRβ contributing to a mature $\alpha\beta$ TCR. The pre-TCR and TCR$\alpha\beta$ are each associated with the *src*-related kinases, *Lck* and *Fyn* (10–12).

Several lines of transgenic and gene knockout mice have indicated that the pre-TCR-dependent DN-ISP-DP transition is inhibited by *Lck* overexpression as well as by *Lck* deficiency or expression of a dominant-negative *Lck* (13–15). Nonetheless, conclusions are inevitably complicated by variability in the onset and durability of *Lck* transgene expression in the different mouse strains and by the uncertain relationship between *Lck* expression and *Lck* activity. Instead, it would be useful to show that different biological effects occur within a single system as *Lck* activity is incrementally altered across a continuous range.

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[4] Abbreviations used in this paper: ASKA, analog-sensitive kinase allele; NaPP, 4-amino-1-*tert*-butyl-3-(1′-naphthyl)pyrazolo[3,4-*d*]pyrimidine; RTOC, reaggregate fetal thymic organ culture; DN, double negative; ISP, immature single positive; DP, double positive; SP, single positive; wt, wild type.

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This seemed an ideal context in which to test the application of ASKA technology to a complex immunological process.

Materials and Methods

Retroviral infections

Lck alleles generated using QuikChange Site-directed mutagenesis (Stratagene, La Jolla, CA) were subcloned into the pMX vector (16). Briefly, 2×10^6 phoenix-E packaging cells were transfected with 20 μg of vector DNA in $CaCl_2$ and 2× HBS (pH 7.05). Medium was changed 8 h after infection, and supernatant viral particles were harvested at 48, 72, and 96 h, passed through 45-μm filters; aliquoted, and stored at −70°C (17).

RTOC, transduction, and formation

Embryonic thymocytes and stromal cells were isolated and prepared as described previously (18). E14 thymocytes were centrifuged at 2100 rpm for 1 h, incubated with virus for 3 h, and pelleted by centrifugation with thymic epithelial cells at a ratio of 1:3. The resultant slurry was transferred by a finely drawn glass pipette to the surface of a 0.8-μm nucleopore filter (19). Cultures were harvested at indicated times and thymocytes were released from reaggregates using fine cataract knives.

Transduction and Western blot of thymoma BW5147

Briefly, 1.2×10^6 cells were transduced with control vector or *Lck^a-as* and, after 48 h, *gfp*$^+$ cells were flow cytometry sorted and expanded in culture with 0, 0.1, 1, 5, or 10 μM NaPP1 (Cellular Genomics, Branford, CT) for another 48 h before lysis in Nonidet P-40 buffer. One hundred micrograms of protein lysate was separated on 10% SDS gels, blotted onto nylon membranes, and probed using Abs against total *Lck* (Santa Cruz Biotechnology, Santa Cruz, CA) and phosphorylated Ysrc416 (Cell Signaling, Beverly, MA), respectively.

Results and Discussion

Lck^a-as *is an active yet inhibitable allele of* Lck

Mutation to glycine of Thr^{316} in the ATP binding site of Lck creates *Lck-as* (Fig. 1*a*), an allele predicted to bind ATP and function as a normal tyrosine kinase (20, 21), but that should also accommodate NaPP1, an enlarged analog of the Src family kinase inhibitor PP1 that competes with ATP for entry into the ATP binding site (3). Other naturally occurring kinases contain a large amino acid corresponding to T316 that precludes NaPP1. Following construction of *Lck-as*, we combined the T316 G mutation in *cis* with a Y505 F mutation (*Lck^a*) that renders *Lck* constitutively active (22), to create *Lck^a-as*

A primary activity of *Lck* is autophosphorylation. To test *Lck^a-as* activity, several T cell lines were transduced with retroviruses expressing *Lck^a-as* from a polycistronic RNA also encoding *gfp* from an internal ribosome entry site. Following transduction, cells were incubated for 48 h in different NaPP1 concentrations, after which equal amounts of *gfp*$^+$ cell lysates were Western blotted and probed with Abs for either total p56^{Lck} (Fig. 1*b*) or Y394-phosphorylated *Lck* (Fig. 1*c*). As the inhibitor was increased, total *Lck* was comparably expressed (serving as a control for protein loading), whereas phosphorylated p56^{Lck} decreased in amount in a dose-dependent fashion. Thus, *Lck^a-as* encodes a novel form of *Lck*, the activity of which is regulatable with NaPP1.

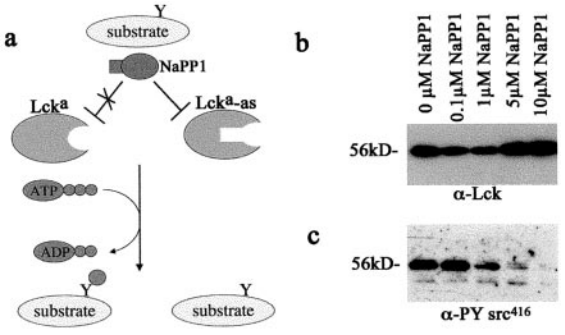

FIGURE 1. a) Schematic representation of inhibition by NaPP1. T316 was exchanged for glycine, creating a unique hole in the ATP-binding pocket of Lck. ATP can normally access the ATP binding site and Lck can phosphorylate downstream substrates. However, in the presence of NaPP1, there is competition for the ATP binding site of the ASKA (*Lck-as*), blocking downstream substrate activation. Nonmutated kinases preclude access to NaPP1. *b* and *c,* BW 5147 thymoma cells were transduced with *Lck^a-as* and *gfp*$^+$ cells were incubated with either 0, 0.1, 1, 5, or 10 μM NaPP1 for 48 h. Western blots were probed with Abs to detect *Lck* (*b*) and Y394-phosphorylated *Lck* (*c*).

Lck^a-as *is biologically active and specifically inhibitable in wild-type (wt) RTOC*

DN thymocytes were transduced with a virus expressing *gfp* only, or coexpressing *Lck^a*, and *Lck^a-as*, respectively, and incubated in RTOC for ~5 days, by which time >50% of *gfp*$^+$ thymocytes transduced with a *gfp*-only virus developed to the DP stage (Fig. 2*a*; in all plots, the *x*-axis depicts CD8 expression, the *y*-axis depicts CD4). Although DP development varied from 50% to 82% in different experiments (e.g., because of age variation in the fetuses used as sources of thymocytes and stroma, or variable time of harvest), results within single experiments were more consistent. Moreover, development was largely comparable in nontransduced cultures, "*gfp*-only" transduced cultures and nontransduced (*gfp*$^-$) cells recovered from RTOC supporting *gfp*$^+$ transduced cells (Fig. 2). Thus, neither transduction nor *gfp* expression substantially affects thymocyte development in RTOC.

Consistent with the deleterious effects of *Lck* overexpression in transgenic mice (15), *gfp*$^+$ thymocytes transduced with an activated Y505 F *Lck* allele (*Lck^a*) showed almost complete inhibition of the DN-DP transition (4% DP), as did *Lck^a-as*-transduced thymocytes (Fig. 2*a*), thereby demonstrating the ASKA to be biologically equivalent to *Lck^a*. However, the biological activity of *Lck^a-as* was substantially inhibited by addition to the RTOC of 10 μM NaPP1. Thus, the inhibitor rescued the generation of DPs (40%) and the expression by many of them of low levels of CD3 (>23% compared with 18% in controls; Fig. 2*a*). The inhibitor's specificity was evident in its failure to rescue RTOCs expressing *Lck^a* (Fig. 2*a*). RTOCs harvested at 9 days also showed strong inhibition of DP maturation by *Lck^a-as* as well as an accumulation of CD8$^+$ ISPs (48%), previously associated with *Lck* overexpression (15). Both effects were substantially reversed by 10 μM NaPP1 (Fig. 2*b*). In sum, the combined use of a novel *Lck* ASKA and an ATP analog inhibitor provided a simple means to alter the activity of *Lck* over a biologically relevant range within a complex organ culture.

The inhibitory effect of activated *Lck* is poorly understood, but it is not readily attributed to apoptosis since transgenic mice expressing increased levels of wt or activated *Lck* showed no obvious decrease in thymocyte numbers (15). Similarly, comparable numbers of *gfp*$^+$ thymocytes were recovered from the *gfp*-only and from the *Lck^a-as*-transduced cultures. The recovery of similar numbers of cells from cultures containing 10 μM NaPP1 indicated that inhibitor per se also does not affect cell viability (see Fig. 2*a*).

To determine whether T cell development shows a graded or an all-or-nothing threshold response to variation in *Lck* activity,

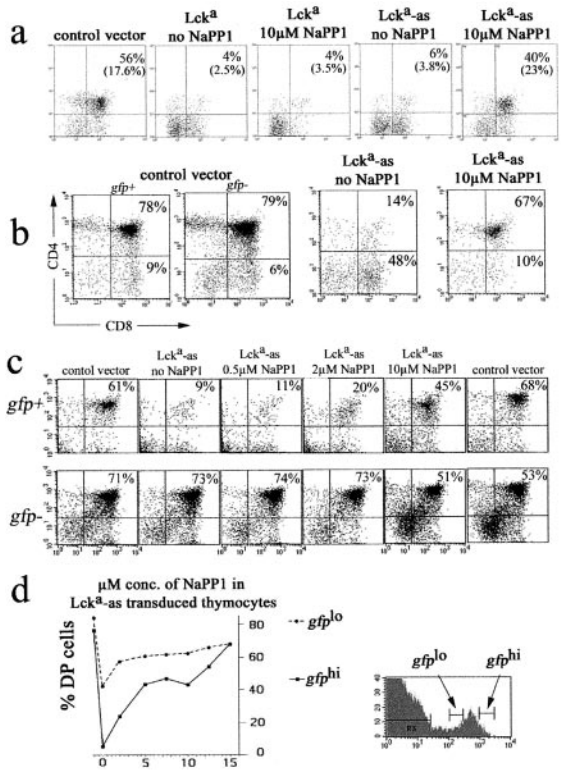

FIGURE 2. *a,* RTOC from wt mice transduced with control (*gfp*-only) vector, *Lck*^{a}, or *Lck*^{a}*-as*, harvested after 5 days with or without 10 μM NaPP1, counted (control: 4.5×10^5 cells; *Lck*^{a} no NaPP1: 4.2×10^5; *Lck*^{a} 10 μM NaPP1: 4.4×10^5; *Lck*^{a}*-as* no NaPP1: 4.1×10^5; *Lck*^{a}*-as* 10 μM NaPP1: 4.2×10^5), and assayed for CD4 (*y*-axis) and CD8 (*x*-axis). Numbers in *upper right quadrants* show the percentage of thymocytes expressing CD4 and CD8; numbers in parentheses show the percentage of CD3^{low} cells. *b,* CD4 and CD8 analysis of RTOC from wt mice transduced with control vector or with *Lck*^{a}*-as*, harvested after 9 days with or without 10 μM NaPP1. Numbers in the *upper right quadrants* show the percentage of DP thymocytes; numbers in *lower right quadrants* show the percentage of ISPs. *c,* CD4 and CD8 expression on thymocytes from wt mice transduced with *Lck*^{a}*-as* and cultured for 5 days in RTOC with indicated concentrations of NaPP1. *Top panels,* Analyses of *gfp*$^+$ cells and *lower panels,* analyses of *gfp*$^-$ cells from the same RTOCs. (cell yields − control: 2.2×10^5; *Lck*^{a}*-as* no NaPP1: 2.4×10^5; *Lck*^{a}*-as* 0.5 μM NaPP1: 1.9×10^5; *Lck*^{a}*-as* 2 μM NaPP1: 3.2×10^5; *Lck*^{a}*-as* 10 μM NaPP1: 2.4×10^5; control: 1.7×10^5 cells). *d,* Percentage of DP cells among *gfp*^{low} vs *gfp*^{high} thymocytes at different inhibitor concentrations. The *left-most points* show the range of DP development (78–82%) in *gfp*-only transduced cultures in this experiment.

different doses of inhibitor were added to RTOCs. In this experiment, the representation of DPs in control vector-transduced cultures ranged from 53 to 71%, whereas for *Lck*^{a}*-as*-transduced cells it was ~9% (Fig. 2*c*). Increasing NaPP1 gradually reduced *Lck* overactivity, incrementally restoring DP cells to 11% (0.5 μM NaPP1), 20% (2 μM), and ~45% (10 μM) (Fig. 2*c*). The development of *gfp*$^-$ cells from the same RTOC was within the range of the controls (Fig. 2*c*) and comparable cell yields again suggested that cell viability was not greatly affected by *Lck*^{a}*-as* or by 10 μM inhibitor (see legends to Figs. 2 and 3). Thus, the dose-dependent regulation of a *Lck* ASKA in RTOC readily exposed a quantitative correlation of T cell development with *Lck* activity across a continuous range.

Fig. 2*d* plots inhibitor concentrations against DP development for *Lck*^{a}*-as*-transduced thymocytes in another RTOC in which cells expressing high or low *gfp* levels were analyzed separately. Since the *Lck* and *gfp* cistrons are linked, most *gfp*^{low} cells express low *Lck* levels, while *gfp*^{high} cells generally express more *Lck*. Consistent with this, comparable DP development (~42%) is seen in *gfp*^{low} cultures with no inhibitor and in *gfp*^{high} cultures with 5 μM inhibitor. However, the enhanced thymocyte maturation, even of *gfp*^{low} cells, at high inhibitor concentrations (~70% DPs) demonstrates that ASKA methodology can reduce *Lck* biological activity to below that achieved by gating for *gfp*^{low} expression. In sum, the application of ASKA technology permits *Lck* activity to be regulated over an unprecedentedly broad, biologically relevant range in a complex organ culture.

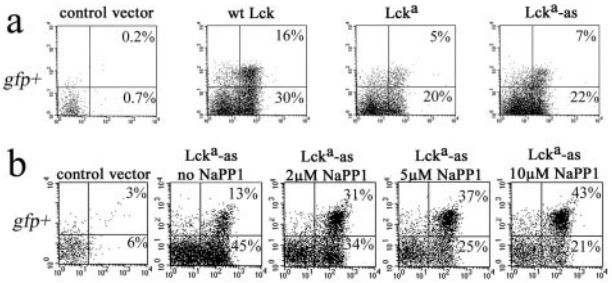

FIGURE 3. *a,* Development of RAG1$^{-/-}$ thymocytes assessed by flow cytometry for CD4 (*y*-axis) and CD8 (*x*-axis) following transduction with wt *Lck*, *Lck*^{a} or *Lck*^{a}*-as*. *b,* CD4 and CD8 expression on *Lck*^{a}*-as*-transduced thymocytes from RAG1$^{-/-}$ mice cultured in indicated concentrations of NaPP1. Number of cells harvested from individual RTOCs: control: 1.5×10^5 cells; *Lck*^{a}*-as* no NaPP1: 1.9×10^5; *Lck*^{a}*-as* 2 μM NaPP1: 1.8×10^5; *Lck*^{a}*-as* 5 μM NaPP1: 1.9×10^5; *Lck*^{a}*-as* 10 μM NaPP1: 1.8×10^5.

Lck^a-as *is biologically active and specifically inhibitable in mutant RTOC*

The biological function of *Lck*^{a}*-as* was independently assayed in RTOC using thymocytes from RAG1-deficient mice that cannot rearrange TCRβ genes and therefore cannot undergo DN-ISP-DP maturation (23). Thus, *gfp*-only vector-transduced thymocytes remain >90% DN (Fig. 3*a*), whereas enforced *Lck* expression mimics pre-TCR signaling, promoting the appearance of ISPs and increasing DP representation from 0.2 to 16% (Fig. 3*a*). Although transgenic *Lck* was reported to rescue DP development more fully than this (24), those studies used a debilitated *Lck*. Instead, *Lck* overexpression in transgenic mice led primarily to ISP accumulation (15). This distortion in thymocyte development was exaggerated in *Lck*^{a} and *Lck*^{a}*-as*-transduced RTOCs in which DP rescue was only 5–7% (Fig. 3*a*). However, increasing doses of inhibitor achieved an incremental rescue of *Lck*^{a}*-as*-transduced RAG1$^{-/-}$ DPs (13, 31, 37, 43%; Fig. 3*b*). Thus, ASKA technology permitted the dose-dependent regulation of thymocyte development by *Lck* in wt and mutant forms of RTOC.

Chemical genetic complementation of Lck *deficiency across a complete range*

Finally, the *Lck*-ASKA was examined for its capacity to complement the defective development of *Lck*$^{-/-}$ thymocytes. *Lck*$^{-/-}$ mice show partial inhibition of the DN-ISP-DP transition (~35% DP cells) because the defect in pre-TCR signaling is partly compensated by *fyn* (Fig. 4*a* and Ref 13). Rather than rescue this deficiency, strong *Lck* overexpression permitted only 1% DPs, consistent with its inhibition of thymocyte development (Fig. 4*d*). However, by selecting for lower *gfp* expression (lower levels of *Lck*), DP representation went from 7%

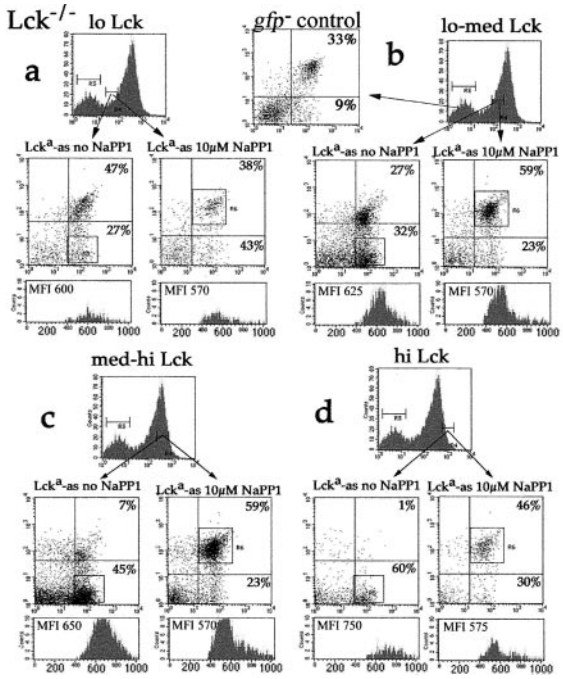

FIGURE 4. CD4 (*y*-axis) and CD8 (*x*-axis) expression on *Lck*^{a}*-as*-transduced *Lck*$^{-/-}$ thymocytes in RTOC cultured with or without 10 μM NaPP1 inhibitor. In each section (*a–d*), the *top panel* shows gating on *gfp* expression (*a*, low; *b*, low-medium; *c*, medium-high; *d*, high); the *middle panel* shows CD4 and CD8 analysis; and the *bottom panel* shows forward scatter fluorescence for gated ISPs (*left panels*) or DPs (*right panels*). The mean fluorescence intensity (MFI) for each analysis is noted. An analysis of *gfp*$^-$ cells from the same culture is shown in the *center* of the *top row*.

(Fig. 4*c*) to 27% (Fig. 4*b*) and to 47% (Fig. 4*a*). However, substantial rescue of DPs (46%) was also achievable among *gfp*^{high} cells, simply by adding 10 μM NaPP1 (Fig. 4*d*). Indeed, within a single NaPP1-treated RTOC, the complementation of *Lck* deficiency was achieved across a full biologic range. Thus, cells gated for "medium" *gfp* in the presence of inhibitor showed essentially complete rescue (~60% DP; Fig. 4, *b* and *c*), whereas *gfp*^{low} cells defined thymocytes in which *Lck* activity was now insufficient, and in which DP representation fell back to 38% (Fig. 4*a*).

Excess Lck *favors cell activation over differentiation*

Although overexpressed *Lck* inhibits TCRβ chain gene rearrangement (25), the inhibition of T cell development in *Lck*-overexpressing mice does not phenocopy RAG deficiency and its underlying nature has not been fully clarified. There is little evidence for excess apoptosis (see above), and it has been noted in transgenic studies and here that there is an associated increase in ISPs (15). The higher the *Lck* expression (*gfp*^{high} and 0 inhibitor; Fig. 4*d*), the greater was ISP accumulation (60%), whereas in the presence of inhibitor, DP representation exceeded ISP representation in all but the *gfp*^{low} cells (Fig. 4). Analysis of forward scatter (Fig. 4) and other properties showed that the ISPs are activated blast cells, whereas DPs are mostly smaller and resting. Of note, as *Lck* activity was gradually increased, the ISPs were more activated, as reflected by increasing forward scatter (600–750; Fig. 4). Therefore, increasing *Lck* activity in early thymocytes appears, at least in part, to skew pre-TCR signaling toward cell activation at the expense of differentiation. This seems consistent with the role of activated *Lck* in malignancy (22). The capacity to sort and analyze cells expressing different levels of *Lck* from ASKA-transduced cells cultured in different inhibitor concentrations now offers the potential to identify protein phosphorylation and/or gene expression patterns that correlate with particular levels of kinase activity and their associated biological outcomes.

In sum, we have combined a novel chemical genetic approach with retroviral transduction of RTOC to achieve rapid and efficient analysis of a kinase playing a crucial role in mammalian cells. Previous study of the dose-dependent effects of *Lck* on T cell development required the labor-intensive generation of multiple lines of transgenic mice expressing different levels of an *Lck* transgene (15). Although those studies provided a firm foundation to validate the use of ASKA as a tool in developmental immunology, it is already clear from Fig. 2*d* that the range of biological effects of *Lck* achievable with ASKA technology exceeds that obtained by selecting cells stochastically expressing different levels of a kinase. Indeed, whereas identification of cell lines or transgenic mice expressing a biologically relevant level of a transgene can be problematic, the capacity to use ASKAs to regulate kinase activity may allow one to use mice or cell lines grossly overexpressing a particular kinase. The relative ease of ASKA construction (successfully applied to v-*Src*, CDK2, *Fus*3, CAMKIIα (4)), the specificity of the inhibitor's effects; the broad range of kinase activity achievable, and the closer correlation of biological outcome with kinase activity, as opposed to inducible levels of gene expression (26, 27) suggest that ASKA technology should be usefully applied to many kinases emerging from the expression profiling of lymphoid cells (28, 29) and from genome sequencing.

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