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Award Number: DAMD17-99-1-9151

TITLE: Murine Models of Breast Cancer: Assessment of the Role of c-Scr in Mammary Tumorigenesis

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REPORT DATE: October 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2003	Annual Summary (1 Oct 2002 - 30 Sep 2003)

4. TITLE AND SUBTITLE
Murine Models of Breast Cancer: Assessment of the Role of c-Src in Mammary Tumorgenesis

5. FUNDING NUMBERS
DAMD17-99-1-9151

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Strong evidence exists that the c-Src non-receptor tyrosine kinase plays a role in the pathology of human breast tumors. The purpose of this study is to examine the role of c-Src in mammary tumorigenesis and elucidate the mechanisms that lead to tumor formation in an animal model for breast cancer. In our previous experiments, we used Src substrates that we cloned to activate c-Src and study its signaling mechanisms. Using one of these substrates, Sin, we characterized a signaling cascade that is activated as a result of Sin binding to Src and Src-mediated Sin phosphorylation, and involves activation of the small GTP-binding protein Rap1. More recently we found that Sin, when phosphorylated by Src kinases, forms a signaling complex consisting of signaling intermediates including the Src related tyrosine kinase Fyn and phospholypase C-γ (PLC-γ). We also found that Sin regulates cell signaling by controlling the activation of PLC-γ, which is important for proliferation in many different cell types. In future experiments, we will use inhibitory RNA oligonucleotides to address the effect of lack of Sin on cellular growth and transformation and we will isolate the Sin protein complex from breast cancer cell lines. These experiments will provide insight into the mechanisms of Src kinase/Sin-mediated tumorigenesis and may lead to the identification of proteins that will be used as targets for drug development.

14. SUBJECT TERMS
Breast cancer, Src, Sin, PLC-gamma

15. NUMBER OF PAGES
76

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

We are studying the role of the non-receptor tyrosine kinase c-Src and its substrates in mammary tumorigenesis in a murine animal model and in cell lines. Our purpose is to use c-Src substrates that we have isolated to gain insight into the molecular interactions that mediate Src-dependent intracellular signaling and transformation. The molecules we developed bind to a conserved regulatory region of c-Src activate Src's enzymatic activity and subsequently act as substrates and effector molecules for c-Src signaling [1]. In our experiments we are focusing on one novel adapter protein that we isolated, Sin, to identify factors which play a role in Src-mediated tumorigenesis in the mammary epithelium. As an adapter molecule, Sin binds to cellular proteins and forms a signaling multiprotein complex [2, 3]. In our experiments we have identified some of the binding partners of Sin, including the Src family member Fyn, PLC-γ and the adapter Crk [3]. We are studying Sin because in our experiments we found that this protein has the unique ability among the other c-Src ligands to activate the transforming potential of c-Src. In addition, the expression of full-length Sin and a truncated Sin mutant was recently shown to be upregulated in mouse mammary tumors. In our future studies we will a) use inhibitory RNA oligonucleotides to inhibit Sin expression and assess the effect of lack of Sin on mammary tumorigenesis. b) Purify the Sin signaling complex to isolate proteins that mediate Sin-dependent c-Src tumorigenesis. These studies are important because they will elucidate the molecular mechanisms that drive mammary tumorigenesis and may lead to the development of strategies to interfere with aberrant Src/substrate activity and carcinogenesis. In addition, these studies may implicate endogenous Sin as a novel regulator of mammary tumorigenesis.

BODY

c-Src is a non-receptor tyrosine kinase that is very important for cellular function [4]. The Src substrate Sin that we have characterized is a multi-adapter molecule that mediates the formation of multi-protein complexes in a phosphotyrosine-dependent manner [1]. Because phosphorylated Sin has the ability to bind to different proteins simultaneously, it also has the potential for activating multiple intracellular pathways with pleiotropic effects on cellular behavior. In our preliminary results we found that a truncated version of Sin, SinΔC, activates two major signaling pathways and the transforming potential of c-Src.

Consistent with this, recent experiments using cDNA microarrays have shown that Sin expression is upregulated in human breast tumors [5]. In addition, we recently identified a variant form of Sin, Sin-MTV (mammary tumor variant) whose expression is upregulated in mouse mammary tumors as compared to normal mammary epithelium. Sin-MTV was identified by screening an expressed sequence tag (EST) database, which was compiled by the National Cancer Institute as part of the Cancer Genome Anatomy Project (CGAP), created to determine the gene expression profiles of normal, pre-cancer, and cancer cells.

The cDNA clone encoding Sin-MTV is similar to that encoding the full-length Sin protein that we previously described ([1]Fig. 1). Full length Sin consists of an N-terminal SH3 domain, a central region with multiple tyrosine containing motifs, two

proline-rich regions that bind to the Src SH3 domain and a conserved C-terminus with unknown function (Fig. 1). In contrast, the Sin-MTV encoding cDNA contains a stop codon that truncates the protein within the substrate binding domain, giving rise to a short protein of 192 amino acids with an apparent molecular weight of ~30kDa (Fig. 1).

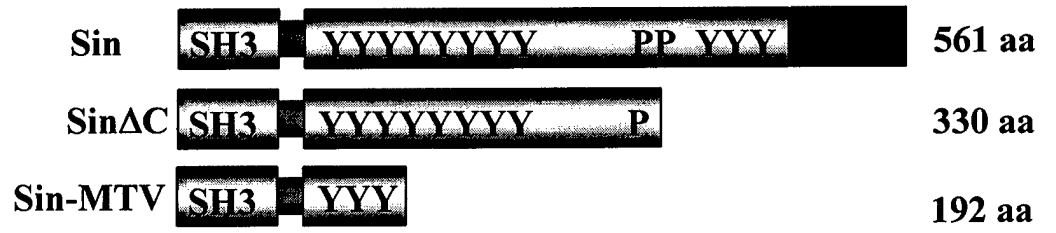

Fig. 1. *Schematic representation of wild type and truncated Sin proteins.* SH3 (Src homology region 3) represent the conserved, proline-binding domain of Sin. Y represents tyrosine containing motifs found in the central, substrate-binding region of Sin. P represents the proline-rich motifs of Sin that interact with the Src SH3 domain.

To address the role of Sin and Sin-MTV in mammary tumorigenesis we obtained cells lines derived from normal mammary epithelium as well as from human mammary tumors and we tested for expression of the Sin proteins. Whereas Sin expression was upregulated in two cell lines we were not able to determine the expression of Sin MTV on western blots. This was most likely due to lack of appropriate antibodies against Sin-MTV. Indeed, Sin-MTV is a truncated mutant and the epitope for the monoclonal antibody we are using is not present on this protein. For this reason we used two new antibodies one against the SH3 domain of Sin and one on the remaining C terminus, but we were still not able to determine with certainty the expression of Sin-MTV in the cell lines we are using. We believe that the failure of these antibodies to bind specifically to Sin-MTV is that SH3 domains from different proteins are highly conserved, and that the C-terminus of Sin is not immunogenic or the antibody epitopes are not exposed when the protein is in its native conformation.

To further examine the role of Sin and Sin-MTV in mammary tumorigenesis and to be able to identify the expression of Sin and Sin-MTV in mammary tumor cell lines, we have been using the recently developed RNA interference (RNAi) [6] technology to down regulate expression of Sin and Sin-MTV in tumor-derived cell lines, and address whether downregulation of Sin expression can reverse the transformed phenotype of these cells. To this end, we generated constructs that express short hairpin RNA (shRNA) molecules that are complementary to different regions of the Sin mRNA. These shRNAs were cloned into a mammalian expression vector, pSURE which allows expression of these shRNA molecules in stably transfected cells. The inhibitory efficacy of these clones was tested in 293HEK cells, lymphocytes, and neuronal PC12 cells but we were not able to downregulate Sin expression. As an alternative we used Sin specific inhibitory RNA oligonucleotides which were transfected directly into 293HEK cells without the use of a plasmid vector. Using this approach we were able to downregulate expression of cotransfected Sin in these cells (Fig. 2). In addition, we are currently developing lentiviral vectors for expressing short inhibitory RNA oligonucleotides that will allow the production of viral particles that can very effectively infect cells and produce RNAi to the gene of interest, in our case Sin and Sin-MTV. Infection of cells

with lentiviral particles will also increase the number of cells that express the inhibitory RNA due to efficient infection of cells, and will allow us to assay the effect of lack of Sin and Sin-MTV in mammary cell transformation. Thus, RNAi oligonucleotides that exhibit an inhibitory effect on Sin expression will be transfected into MCF-7 and SKBR-3 transformed cell lines to assess the effect of Sin downregulation on tumorigenesis. If Sin is required for tumorigenesis, inhibition of Sin expression should reverse the transformed phenotype.

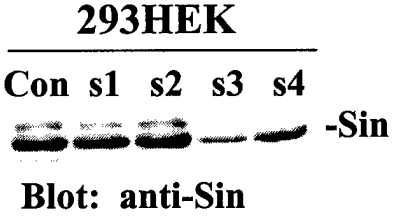

Fig. 2. 293HEK cells were transfected with control (con) or Sin-specific (s1-4) RNA oligonucleotides along with a plasmid expressing full-length Sin. Cells were incubated for 24 hrs, then lysed and western blots of cell extracts were probed with specific antibody. Two of the Sin inhibitory oligonycleotides (s3 and s4) were found to inhibit Sin expression.

In aim 2 of the original application we proposed to use conserved sequences of Sin to identify elements that are important for the signaling properties of the protein and use these sequences as probes to identify proteins that mediate Sin-dependent Src signaling. Thus far we have characterized the signaling mechanisms of Sin and identified proteins some of the proteins that are involved in mediating Src signaling using the human embryonic carcinoma 293HEK cell-culture system [2]. In this study, through the use of mutagenesis analysis and dominant negative inhibitors, we found that Src phosphorylated Sin forms a signaling complex that consists of the adapter protein Crk and C3G, a nucleotide exchange factor that promotes guanyl-triphosphate (GTP) binding on the GTP-binding protein Rap1.

In parallel experiments we aimed to address the in vivo role of Sin using organs of the immune system (thymus and spleen) as model tissues to address the biological function of Sin. These experiments are relevant since Sin is highly expressed in the thymus and to a lesser extent in the spleen. We found that in these cells Sin forms a multiprotein complex with other signaling molecules of which we identified the Src-related kinase Fyn and phospholypase-C-γ (PLC-γ) [3, 7]. Association of the Crk and C3G proteins with Sin was also observed in T cells, as well as Rap1 activation [2, 8]. We also found that Sin regulates the phosphorylation and activation of PLC-γ and as a consequence intracellular calcium release, MAP kinase activation and T cell proliferation [3]. Activation of PLC-g and intracellular calcium release are important signaling events that mediate transcriptional activation and proliferation of many different cell types.

Given that Sin associates with other proteins and forms a multiprotein signaling complex that regulates cellular function, we believe that isolation of this complex will provide important insight into the function of Sin an its effect on cellular behavior. To this end we have generated a Sin construct that expresses full-length Sin fused to a small peptide comprising the flag epitope, which allows the purification of proteins that contain

it over anti-flag-specific affinity columns. To purify proteins that may be relevant for Sin function in the mammary epithelium the flag-Sin construct will be expressed either transiently or stably in mammary tumor cell lines. Large-scale purification of flag-tagged Sin and associated proteins will then be performed over anti-flag columns, and acid eluted complexes will be separated by SDS-PAGE. Protein bands will be excised from Coomassie-stained SDS-PAGE gels and digested with trypsin. Extracted peptides will then be subjected to matrix-assisted laser (MALDI) or electrospray ionization (ESI) peptide mapping on a QSTAR XL mass spectrometer in the core spectrometry facility of Columbia University. Resulting peptide sequence data will be used to search the NCBI non-redundant protein database to identify proteins associated with Sin. We believe that this newly-developed purification approach is mote efficient in isolating proteins bound to Sin that using isolated Sin conserved sequences, since this approach will identify the entire Sin signaling complex. We anticipate that Sin complex purification will identify both known and novel proteins that associate with Sin and mediate/regulate its function in mammary epithelial cells, including the proteins mentioned above that we already identified as Sin binding proteins.

KEY RESEARCH ACCOMPLISHMENTS

1. We have shown that Sin interaction with Src leads to the activation of Src signaling as assayed by transcriptional activation.
2. We have for the first time described a novel pathway that mediates wild type c-Src signaling.
3. Our experiments have revealed mechanistic differences in the signaling mechanisms of wild type versus transforming Src alleles.
4. We have confirmed that this pathway operates in vivo downstream of Src kinases using thymocytes and T cells as a model system.
5. We have characterized the components of this pathway both in vitro and in vivo. These include Src/Sin, the signaling complex Crk/C3G, the G-protein Rap1 and the kinase ERK1.
6. We have found that the Src kinase Fyn is important for Sin phosphorylation in T lymphocytes and that Fyn mediates some of the inhibitory effects of Sin on T lymphocyte function.
7. We have also found that Sin is constitutively phosphorylated and bound to substrates in resting cells. These substrates include the Src-related kinase Fyn and PLC-γ. In addition, our data show that Sin regulates the activation of PLC-γ and as a consequence intracellular signaling events important for cell proliferation.

REPORTABLE OUTCOME

-We have published the following manuscripts:

1. Xing L., Ge, C, Zeltser R, Maskevitch GR, Mayer, BJ, and **Alexandropoulos K.** 2000. c-Src signaling induced by the adapters Sin and Cas is mediated by the Rap1 GTPase. *Mol. Cell. Biol.* 20:7363-7377.

2. Donlin, L.T., Roman, C.A., Adlam, M., Regelmann, A.G., and **Alexandropoulos, K.** 2002. Defective thymocyte maturation by transgenic expression of a truncated form of the lymphocyte adapter molecule and Fyn substrate, Sin. *J. Immunol.* 169:6900-6909.

3. **Alexandropoulos, K.,** Donlin, L.T., Xing, L. and Regelmann, A.G. 2003. Sin: Good or Bad? A T lymphocyte perspective. *Immonol. Rev.* 192: 181-195.

4. Xing, L., Donlin, L.T., Miller, R.H., and **Alexandropoulos, K.** 2003. The Adapter Molecule Sin Regulates T-Cell-Receptor-Mediated Signal Transduction by Modulating Signaling Substrate Availability. *Mol. Cell. Biol.* Submitted (under revision).

-Oral presentations and posters at scientific meetings:

An oral presentation and an abstract were presented in an annual meeting: "Tyrosine Phosphorylation and Cell Signaling: The Third Decade". August 9-13, 2000, The Salk Institute, San Diego, CA.

An oral presentation was given at the FASEB annual meeting: "FASEB 2000, Signal Transduction in the Immune System". Saxton River, VT.

Two abstracts were presented at "The Fortieth Midwinter Conference of Immunologists: Immune System Development and Function" , January 27-30, 2001, Asilomar, CA

An oral presentation was presented at the International Symposium on: "The Molecular Basis of Immune Cell Activation and Immunological Disorders", February 15-18, 2001, San Diego, CA

An oral presentation was presented at the "Receptors and Signal Transduction" meeting, June 29-July-3, 2002, Salt Lake City, Utah

A poster was presented at the "Era of Hope" meeting, September 25-28, 2002, Orlando, FL
An oral presentation was presented at the "2nd Lymphocyte Signal Transduction Workshop" October 13-17, 2002, Santorini, Greece.

-No degrees obtained

-We have developed cell lines coexpressing Src and SinΔC using NIH3T3 cells.

-No informatics

-We have received funding from the National Institute of Allergy and Infectious Diseases (NIAID) on work supported by this award

RO1 AI49387 $1,125,000
Title: Examine the Role of Fyn and Rap1 in T cell activation and T cell-mediated Immune Responses.

-No changes in employment status.

CONCLUSIONS

Oncogenic Src proteins have been extensively studied to gain insight into the signaling mechanisms of Src. To better understand signaling through wild-type Src, we cloned a substrate, Sin, which activates Src signaling by binding to the Src SH3 domain. To this end, we used full length and truncated versions of Sin to activate c-Src, and examined the intracellular pathways that mediate Src signaling under these conditions. We found that Sin-induced Src signaling is exclusively mediated through a pathway that consists of the Crk/C3G/Rap1 signaling complex. The involvement of this pathway downstream of Src kinase-phosphorylated SinΔC was confirmed in vivo using thymocytes and T cells as model systems. Our current experiments, described above, are aimed towards addressing the role of Sin in mammary tumorigenesis, either independently or in conjunction to Src, as well as the mechanisms through which Sin may exerts its tumorigenic effects. These experiments are important in that, they may reveal an important function for Sin in mammary tumorigenesis, as well as identify novel molecules that mediate Sin function. These molecules in turn may serve as putative therapeutic targets for treating human breast tumors.

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APPENDIX

The Adapter Molecule Sin Regulates T-Cell-Receptor-Mediated Signal Transduction by Modulating Signaling Substrate Availability

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Running title: The adapter Sin inhibits T cell activation

Key words: Src kinase, PLC-γ, Fyn, T cell, signaling

Word count: Materials and Methods: 932, Introduction/Results/discussion: 4,226

ABSTRACT

Engagement of the T cell receptor (TCR) results in the activation of a multitude of signaling events that regulate the function of T lymphocytes. These signaling events are in turn modulated by adapter molecules, which control the final functional output through the formation of multi-protein complexes. In this report, we identified the adapter molecule Sin as a new negative regulator of T cell activation. We found that expression of Sin in transgenic T lymphocytes and Jurkat T cells inhibited interleukin-2 (IL-2) expression and T cell proliferation. This inhibitory effect was specific and was due to defective phospholipase-C-γ (PLC-γ) phosphorylation and activation. In contrast to other adapters that become phosphorylated upon TCR stimulation, Sin was constitutively phosphorylated in resting cells by the Src kinase Fyn and bound to signaling intermediates including PLC-γ. In stimulated cells, Sin was transiently dephosphorylated, which coincided with transient dissociation of Fyn and PLC-γ. Similar results were observed in Sin-expressing Jurkat T cells and normal primary T lymphocytes, suggesting that these molecular events are physiologically relevant. We conclude that endogenous Sin influences T lymphocyte signaling by regulating the availability and/or activation of signaling intermediates in resting cells, while it may act to increase the local concentration of signaling molecules for fast signal transmission during stimulation.

INTRODUCTION

In recent years, adapter molecules have emerged as critical regulators of intracellular signaling pathways and function in T cells. While these molecules lack intrinsic enzymatic activity, they regulate and integrate signaling events through protein-protein and protein-lipid interactions. T cells express a variety of adapter molecules that are classified as positive or negative regulators of TCR signaling. Positive regulators of T cell function include the bona fide adapters Grb-2 and Gads, as well as the scaffold proteins Src homology 2 domain containing leukocyte protein-76 (SLP-76), linker of activation of T cells (LAT), and degranulation-promoting adapter protein (ADAP), whereas negative regulators consist of the Cbl family of proteins, SLAP (Src-like adapter protein), and protein associated with glycosphingolipid-enriched microdomains PAG/Cbp (18, 25, 36).

Gene targeting experiments in Jurkat T cell lines and mice indicate that deletion of positive regulators in general, exhibit defective thymocyte development or T cell signaling (8, 29, 30, 39, 42), whereas mice lacking negative regulators have opposite phenotypes manifested as increased thymocyte positive selection and development of autoimmunity (22, 24, 33). Similarly, studies describing overexpression of adapter molecules in T lymphocytes have helped to further define these molecules and have shown that negative and positive regulators either suppress or promote excessive T lymphocyte development and function, respectively (19, 34, 35, 37).

Our studies are concentrated on elucidating the molecular mechanisms involved in TCR signaling, with particular emphasis on the role of adapter/scaffold molecules in this process. More specifically, we are interested in examining the role of the novel

adapter molecule Sin in T cell signaling and activation. Sin belongs to a small family of related proteins, the other members being p130Cas and Cas-L (27). These proteins share a conserved Src homology region 3 (SH3) domain, repeated tyrosine-based and proline-rich motifs, and a conserved C terminus. All three members of the p130Cas family are substrates for Src kinases and Src kinase-mediated phosphorylation of these proteins is important for their adapter/scaffold signaling properties (2).

We became interested in determining whether Sin regulates TCR signaling because Sin was cloned as a substrate for the key TCR signaling molecule Fyn (1, 13), and because the thymus expresses higher levels of Sin in comparison to other tissues (2, 12). Thus, in previous experiments we examined the role of Sin in thymocyte development using transgenic mice expressing a truncated form of Sin, SinΔC. SinΔC expression in the mouse thymus resulted in reduced thymic cellularity due to increased thymocyte apoptosis, as well as defective thymocyte differentiation manifested as reduced numbers of mature $CD4^+$ and $CD8^+$ single positive (SP) cells (6). We also found that the Src kinase Fyn was important for Sin-mediated thymocyte apoptosis but not for the inhibition of thymocyte maturation (6). These results suggest that Sin is a negative regulator of thymocyte differentiation and survival.

In this report we addressed the role of Sin in TCR signaling and T cell activation using Jurkat and transgenic T cells expressing full-length Sin or SinΔC respectively. We found that Sin expression inhibited TCR-induced T cell activation and proliferation by blocking expression of the IL-2 gene. The defect in IL-2 expression correlated with reduced PLC-γ phosphorylation, intracellular calcium release, and NFAT and AP-1 activation. Strikingly, we found that Sin was constitutively phosphorylated in resting

cells, which correlated with association of phosphorylated Sin with multiple signaling molecules of which we identified Fyn and PLC-γ. This adapter function of Sin was modulated through the TCR, because Sin was dephosphorylated after TCR stimulation, which coincided with the release of proteins bound to Sin in resting cells including Fyn and PLC-γ. Thus, these data suggest that Sin is a negative regulator of T cell activation and that Sin influences T lymphocyte signaling by regulating the availability and/or activation of signaling substrates, thus controlling IL-2 production and T cell proliferation.

MATERIALS AND METHODS

Mice. A cDNA fragment encoding amino acids 1-335 of full length Sin was cloned into the EcoRI/SmaI site of the CD2 expression cassette (43) and SinΔC transgenic mice were generated as previously described (6). C57BL/6 animals were purchased from the Jackson Laboratory (Bar Harbor, Maine).

cDNA constructs. DNA manipulations were performed by standard protocols. Full length Sin, Efs2 (missing amino acids 4-99), and SinΔC (amino acids 1-335), were cloned into the *SpeI-NotI* sites of the pEBB expression vector. The pEBB was derived from the pEF-BOS expression vector driven by the human elongation factor 1-α promoter (20).

Cell lines and antibodies. Jurkat cells transfected with the Simian virus 40 large T antigen (SV40 Tag, provided by A. Weiss, University of California at San Francisco, San Francisco, CA), the parental Jurkat E6-1, and primary splenic T cells were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin, streptomycin and glutamine. Stable cells lines were generated by cotransfecting $2x10^6$ Jurkat TAg

cells with 1μg pEBB-Sin and 0.2μg MSC-puro vector expressing puromycin using the FuGENE-6 transfection reagent (Roche). Transfected cells were serially diluted in 96 well plates and selected in culture medium containing 300ng/ml puromycin.

Mouse monoclonal antibodies: anti-Sin-specific and isotype-matched control MOPC were obtained from BD Transduction Laboratories; anti-Fyn, anti-Lck, and PLC-γ (clone sc-81) were obtained from Santa Cruz; anti-phosphotyrosine-specific antibody from Upstate Biotechnology. Purified mouse monoclonal anti-CD3 and CD28 antibodies used for crosslinking were obtained from BD Pharmigen.

Flow cytometry. $1x10^6$ freshly isolated splenocytes were incubated with the appropriate antibodies in staining medium (3% Fetal Calf Serum, 0.1% Sodium Azide in PBS) for 15 min on ice. Cells were spun down and washed three times with staining medium and analyzed by flow cytometry using a FACS Calibur and CELLQUEST software. Anti- CD4-Allophycocyanin (APC), CD8-Peridinin Chlorophyll-a Protein (PerCP), and CD3-Fluorescein Isothiocyanate (FITC) conjugated antibodies were purchased from BD Pharmigen.

T cell purification and proliferation assays. Splenic $CD4^+$ T cells were purified using the Dynabead/DETACHaBEAD Mouse $CD4^+$ system (DYNAL Biotech). 1×10^5 T cells were plated per well of a 96-well plate. Cells were left untreated or induced with plate bound mouse anti-CD3ε (0.5μg/mL) and anti-CD28 (5μg/mL) or anti-CD3ε (0.05μg/mL) with or without recombinant mouse IL-2 (20ng/mL) (BD Pharmingen). At 72 hours, cells were labeled overnight with 1 μCi [^{3}H]thymidine and the proliferative response was determined by levels of [^{3}H]thymidine incorporation on a scintillation

counter. 5μl of supernatant from each well in the proliferation assay was collected at 24, 48, 72 hours and assayed for IL-2 concentration (R&D Systems mouse IL-2 ELISA kit).

Transfections and luciferase assays. For luciferase reporter assays $2x10^6$ Jurkat TAg cells were transfected with the indicated amounts of plasmid DNA expressing Sin, SinΔC, Efs2, or SLP-76 proteins plus 200ng NFAT-or AP-1-Firefly-Luciferase and 5ng pRL-TK-Renilla-Luciferase reporter constructs using FuGENE-6 transfection reagent according to manufacturer's protocol. Expression of the Renilla luciferase is under the influence of the Herpes Simplex Virus thymidine kinase (HSV-TK) promoter (Promega). After overnight incubation, half of the transfected cells were stimulated either by plate-bound OKT3 antibody (5μg/ml), or PMA (10ng/ml) plus Ionomycin (1μM) for 8 hours. Induced and uninduced cells were lysed in 50μl lysis buffer and 20μl cell lysates were assayed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Measurement of intracellular calcium. Cells at a concentration of 4×10^6 were incubated with 1μM indo-1/AM for 1 hour at 37 ^{0}C in RPMI-1640 plus 1% FBS. Cells were washed three times in buffer A (10mM HEPES, pH7.4, 3mM KCL, 1mM $MgCl_2$, 1mM $CaCl_2$, 140mM NaCl, 0.1% glucose, 1% FBS) and suspended at a final concentration of 1×10^6 in buffer A. Cells were stimulated with 1μg/ml OKT3. The fluorescence ration (405nm/485nm) with excitation at 350nm was obtained from a spectrofluorometer (Photo Technology Internaltional, Lawrenceville, NJ 08645). Ionomycin was added to a final concentration of 15μM in order to obtain maximum fluorescence under saturating calcium concentrations, followed by 10mM EGTA to

obtain minimum fluorescence in the absence of calcium. Calibrations and calculation of intracellular calcium concentration were conducted as previously described (9, 31).

Analysis of IP3 Release. $4x10^6$ cells were induced with soluble OKT3 antibody (1μg/ml) for 3 minutes and cell extracts were generated by adding 80μl ice-cold 100% Trichloroacetic acid. IP3 levels were assessed using the IP3 Radioreceptor Assay Kit (NEN Life Science Products, Boston, MA) following the manufacturer's protocol.

TCR crosslinking. 1×10^7 splenocytes were incubated with 2μg anti-CD3 and CD28 antibodies on ice for 15 min, washed with cold phosphate buffered saline (PBS) and super-crosslinked with 5μg of goat-anti-mouse IgG for 20 min on ice. Cells were then incubated at 37 ^{0}C for different points, spun down and immediately lysed. Cell lysates were used for immunoprecipitation and immunoblot analysis.

Immunoprecipitations. Immunoprecipitations were performed as previously described (1). Briefly, cells were lysed in 1ml of ice-cold NP-40 lysis buffer [1% NP-40, 20mM Tris-HCl (pH 8.0), 150mM NaCl, 10% glycerol, 10mM NaF, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 10μg/ml aprotinin, 10μg/ml of leupeptin]. Cell extracts were then incubated with the specified antibodies at concentrations suggested by the manufacturers for 2 hr at 4^0C, and the immune complexes were subjected to SDS-PAGE electrophoresis and immunoblotting. Western blots were performed as previously described (38).

In vitro kinase assays. Protein complexes obtained by immunoprecipitation were washed three times in kinase buffer and reactions were carried out in 20μl of kinase buffer containing 20mM HEPES pH 7.4, 5mM MnCl, 10μM ATP and 1 μl of [γ-^{32}P] ATP (5000Ci/mmole) at room temperature for 10 min. The pellets were resuspended in

1X Laemli buffer, boiled for 2 min and phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

RESULTS

Two Sin isoforms are expressed in T cells. At least two isoforms of Sin can be detected in mouse primary T cells: full-length Sin and the previously described Efs2, a Sin alternative splice form in which the SH3 domain is deleted (12) (Fig. 1A and B). A third protein band, Sin III, is also detected in Sin immunoprecipitates migrating slightly faster than Efs2, and may correspond to a different Sin isoform (2). In Jurkat SV40 Tag cells and the parental line Jurkat E6-1, Efs2 is the predominant form of Sin being expressed (Fig. 1C).

As mentioned above, we previously used transgenic mice expressing a truncated form of Sin, SinΔC, to explore the physiologic role of Sin in vivo (6). This truncated Sin mutant was chosen based on the finding that it enhanced Src kinase signaling greater than the full-length protein (38). Two founder transgenic lines, CR1 and MA2, were generated expressing approximately 27 and 9 fold SinΔC respectively, as compared to the endogenous Efs2 isoform (6). The expression of SinΔC was regulated by the human CD2 promoter, which allows expression of cloned genes in both thymocytes and mature T cells (43). Expression of the SinΔC transgene was demonstrated in splenocytes from two different founder mice (Fig. 1D).

SinΔC expression reduces numbers of peripheral T cells. We previously found that SinΔC expression inhibited thymocyte development manifested as reduced percentages of mature $CD4^+$ and $CD8^+$ SP cells in the thymus and increased thymocyte apoptosis suggesting a negative function for SinΔC in T lymphocytes (6). Here, we used

the SinΔC mice to examine the effect of SinΔC on mature T cell activation and proliferation. First we examined the effect of SinΔC protein expression on the production of mature T cells in the spleen. Fluorescence Activated Cell Sorting (FACS) analysis of total splenocytes revealed that the percentages of mature splenic T cells of both the $CD4^+$ and $CD8^+$ lineages were substantially reduced in transgenic CR1 and MA2 animals as compared to normal littermate controls (Fig. 2A, dot plots). Nevertheless, splenic T cells from transgenic animals expressed normal levels of CD3ε as compared to wild type controls (Fig. 2A, histograms). Consistent with this, we previously found that in addition to normal TCR levels, the expression levels of maturation markers such as CD69 and CD5 in thymic T cells were also normal (6). This suggests that although the percentages of cells are reduced, the existing mature T cells have undergone positive selection. Analysis of several MA2 SinΔC animals further revealed a consistently dramatic decrease in total $CD3^+$ T cell numbers as well as $CD4^+$ and $CD8^+$ mature T cells (Fig. 2B). Relative proportions of T and B cell in the spleen, determined by Thy1.2 and B220 staining, also revealed a dramatic decrease in T cell numbers, whereas the B cell compartment was normal (data not shown). Thus, our data show that SinΔC expression results in reduced numbers of $CD4^+$ and $CD8^+$ SP cells in the spleen of transgenic animals, consistent with T cell-specific expression of SinΔC from the CD2 promoter.

SinΔC expression inhibits T cell activation. We next tested whether SinΔC expressing T cells were able to respond to stimulation through their TCR. Equal numbers of T cells purified from the spleens of normal and SinΔC transgenic animals (CR1 and MA2) were stimulated with plate-bound CD3- and CD28-specific antibodies and PMA/Ionomycin as shown in Fig. 3. As indicated above (Fig. 2A, histograms), TCR

expression levels were similar in purified normal and SinΔC-expressing cells assayed by CD3 staining and flow cytometry (data not shown). We found that SinΔC expressing T cells stimulated through their TCR with CD3, CD3 and CD28 or PMA exhibited dramatically reduced proliferation as compared to normal controls (Fig. 3A). In contrast, transgenic T cells stimulated with PMA and ionomycin, a non-physiologic stimulus that bypasses the TCR, proliferated with the same efficiency as their wild type counterparts, suggesting that the inhibitory effect of SinΔC on T cell proliferation is specific to proximal TCR signaling events (Fig. 3A, bottom graph).

Upon stimulation, T cells secrete the critical cytokine inteleukin-2 (IL-2), which acts in an autocrine fashion to stimulate T cell proliferation. We found that reduced proliferation of SinΔC-expressing T cells was accompanied by a significant reduction in the level of secreted IL-2 suggesting defective TCR signaling leading to cytokine production (Fig. 3B, top graph). The addition of exogenous IL-2, however, restored the proliferative response of the transgenic T cells (Fig. 3B, bottom graph), indicating that signaling events downstream of the cytokine receptor remained intact. Consistent with this, upregulation of the IL-2 receptor (CD25) was normal in transgenic cells stimulated with anti-CD3/CD28 as compared to liter-mate controls (data not shown). These results suggest that the inability of SinΔC-expressing T cells to proliferate is specific to the TCR and is due to defective TCR-mediated IL-2 production and not to an inherent inability of these cells to respond to stimuli.

Full length Sin expression inhibits TCR-mediated transcriptional activation in human Jurkat cells. The results presented above suggest that SinΔC is a negative regulator of T cell activation by rendering cells incapable of IL-2 production. The

mechanism of SinΔC-mediated inhibition of IL-2 is currently not known. Therefore, to further analyze the inhibitory effect of Sin on TCR signaling and to examine whether SinΔC mimics the function of Sin we used transiently or stably transfected human Jurkat T cells. Jurkat Tag cells were transfected with increasing concentrations of a mammalian vector expressing Sin along with two reporter plasmids expressing two different luciferase proteins: one from firefly under three copies of the distal NFAT/AP-1 or multimerized AP-1 binding sites from the IL-2 promoter (10, 32); the other from the sea pansy Renilla under the herpes simplex virus (HSV) thymidine kinase (TK) promoter, which was included as an internal control for transfection efficiency. We found that expression of Sin in Jurkat cells efficiently inhibited NFAT promoter activation in response to TCR stimulation in a concentration dependent manner (Fig. 4A, top graph). In contrast, in the same experiments activation of the Renilla luciferase reporter (HSV promoter) remained constant, suggesting that the inhibition we observe is not because of cell toxicity due to Sin overexpression (Fig. 4A, bottom graph). Importantly, the inhibitory effect of Sin was evident even when expressed at levels similar to endogenous Efs2, arguing against a negative effect of Sin due to gross overexpression (Fig. 4A, inset).

To further address whether the inhibitory effect on NFAT activation is specific to Sin we also examined the effect of other adapter molecules on T cell activation. To this end we used SLP-76, an established positive regulator of T cell function (23, 30), as well as CasL, a Sin related protein also shown to stimulate T cell signaling (16, 26). In contrast to Sin, we found that expression of the Sin related molecule CasL (Fig. 4B), or SLP-76 (Fig. 4C), stimulated NFAT activation in response to TCR crosslinking. These results

suggest that the effects of Sin are specific and that Sin represses TCR-induced transcriptional activity that regulates IL-2 expression.

In addition to NFAT, we also examined the effect of Sin on AP-1 activation, which is a complex of Fos and Jun transcription factors required for IL-2 gene expression. Similarly to NFAT, we found that Sin expression also inhibited AP-1 dependent transcriptional activation in response to TCR stimulation (Fig. 4D, top graph), but not in response to stimulation with PMA/Ionomycin (Fig. 4D, bottom graph). These results suggest that the inhibitory effect of Sin is specific to TCR signaling and that this effect can be bypassed by non-physiologic stimuli.

These and previous data suggest that Sin, as well as the truncated SinΔC form, act as negative regulators of TCR signaling. To address whether SinΔC mimics the function of Sin, we directly compared the ability of these proteins, as well as the Efs2 isoform, to inhibit T cell signaling in Jurkat T cells (Fig. 4E). We found that all proteins share the same inhibitory function, although SinΔC was somewhat less potent in inhibiting transcription than Sin or Efs2 (Fig. 4E) when the proteins were expressed at similar levels (Fig. 4A, insert). These results suggest that the C terminus of Sin contributes to the maximal inhibition of signaling observed with the full-length protein, but is not by itself sufficient for complete inhibition of signaling, since a significant block of NFAT activation is observed with SinΔC, which lacks this region. Thus, SinΔC behaves similarly to Sin and acts as a negative regulator of TCR signaling.

Sin expression inhibits PLC-γ phosphorylation and activation. We next examined the mechanism of Sin-mediated inhibition of transcriptional activation by examining upstream signaling events that control activation of the NFAT and AP-1

transcription factors. It has been established that TCR-induced PLCγ phosphorylation and activation leads to release of the second messengers inositol 3-phosphate (IP3) and diacyl-glycerol (DAG). The production of IP3 induces intracellular calcium release and NFAT activation, whereas DAG controls AP-1 activation through the stimulation of protein kinase C (PKC-θ) and Ras-GRP (11, 18, 36). We thus examined whether these signaling events were compromised by Sin expression using Jurkat T cell lines stably overexpressing Sin. Consistent with the results obtained from transient assays we found that expression of Sin blocked NFAT-luciferase activation in response to TCR stimulation in three different cell lines (Fig. 5A).

Using these cell lines we then examined the effect of Sin expression on PLC-γ phosphorylation and activation. We found that TCR-mediated PLCγ phosphorylation was reduced in Sin-expressing Jurkat T cells as compared to control cells transfected with vector alone (Fig. 5B). Consistent with decreased phosphorylation, we found that the enzymatic activity of PLC-γ was also reduced in T cells expressing Sin shown by reduced levels of IP3 production (Fig. 5C). As a result, TCR-stimulated intracellular calcium release was also compromised in these Sin-expressing cell lines as compared to cells stably transfected with vector alone (Fig. 5D). These results suggest that the defective activation of NFAT/AP1-mediated transcription in Sin-expressing T cells is the result of defective PLCγ phosphorylation/activation and calcium release. These results also show that Sin inhibits T cell signaling by exerting its effects on signaling events proximal to the TCR, which regulate phosphorylation and activation of PLCγ.

To further analyze the inhibitory effects of Sin expression on PLCγ activation, we examined whether Sin directly interacts with PLC-γ in the Sin-expressing cell lines.

Surprisingly, we found that Sin was constitutively associated with endogenous PLC-γ in resting T cells and this association was disrupted in stimulated cells (Fig. 5E). Thus, PLCγ activation correlated with its disassociation from Sin after TCR stimulation. Ten minutes after stimulation, the association of Sin with PLCγ was once again evident, coinciding with downregulation of TCR signaling (Fig. 5E). To address the physiological significance of the Sin/PLC-γ interaction, we also tested the association of endogenous Sin and PLCγ in primary T cells. Importantly, and similar to our results with Jurkat T cells we found that endogenous Sin was constitutively associated with endogenous PLCγ in resting primary T cells, but less so in cells stimulated with anti-CD3/CD28 for 3 minutes (Fig. 5F). These results suggest that Sin preferentially binds to the non-activated form of PLCγ and that it may limit its availability and/or activation in unstimulated cells under physiologic conditions. Overexpression of Sin reveals this function and results in inhibition of PLCγ phosphorylation and activation.

The adapter function of Sin is regulated by constitutive phosphorylation and TCR induced dephosphorylation. Given that TCR signaling molecules typically experience an increase in their tyrosine phosphorylation content after TCR induction, we sought to determine if Sin also underwent such modification. The majority of adapter proteins that regulate TCR signaling, such as LAT, SLP-76, and Cbl, are rapidly phosphorylated after T cell receptor crosslinking under endogenous or overexpression conditions (7, 21, 41). In stark contrast, we found that Sin was constitutively phosphorylated in resting cells and was rapidly dephosphorylated after TCR stimulation in total cell extracts and in Sin immunoprecipitates (Fig. 6A). The basal constitutive phosphorylation of Sin returned after 10 minutes of TCR stimulation, which coincided

with a reduction in total cellular phosphotyrosine levels (Fig. 6A, left panels). The dephosphorylation of Sin after TCR stimulation is likely due to phosphatase activity considering the rapid rate of dephosphorylation (2 min). Thus, Sin is regulated differently from other signaling phosphoproteins, as Sin becomes dephosphorylated rather than phosphorylated upon TCR stimulation.

To further characterize the regulation of Sin phosphorylation and to determine if the pattern of phosphorylation for overexpressed Sin reflects that of endogenous Sin, we performed *in vitro* kinase assays using immune complexes precipitated with Sin-specific antibody. In these assays we sought to compare the levels of kinase activity associated with Sin under resting and TCR stimulated conditions. In Sin immunoprecipitates from Jurkat T cells, we found that both overexpressed full-length Sin and the endogenous Efs2 isoform were phosphorylated *in vitro* in unstimulated Jurkat T cells by an associated kinase(s) (Fig. 6B, left and middle panels). In addition, we observed that Sin associated with several proteins that became phosphorylated *in vitro* in unstimulated cells (Fig. 6B, left and middle panels). TCR stimulation disrupted the *in vitro* phosphorylation of Sin as well as that of associated substrates suggesting either decreased kinase activity or dissociation of a kinase(s). The effects we observed were specific as no phosphoproteins were detected from precipitates of isotype-matched control IgG (Fig. 6B, right panel). Similar results were obtained with endogenous Sin when primary mouse thymocyte cell extracts were used for the kinase assays (Fig. 6C). This suggests that a similar mode of regulation exists for phosphorylation of overexpressed Sin in T cell lines and endogenous Sin in primary T lymphocytes, such that Sin is phosphorylated in resting cells and TCR stimulation results in a loss of associated kinase activity.

The constitutive phosphorylation of Sin on tyrosine residues in resting Jurkat cells suggested a constitutive interaction with a protein tyrosine kinase. Sin was identified as a ligand for Src kinase SH3 domains and more recently, we found that in thymocytes the truncated form of Sin, SinΔC, also binds to and is constitutively phosphorylated by Fyn but not the related kinase Lck (6). Thus, we tested the association of Sin with Fyn in transiently transfected Jurkat T cells. Consistent with the above data, we found that transiently transfected Sin was constitutively phosphorylated in resting cells and became dephosphorylated after 2 minutes of stimulation (Fig. 6D, left panels). Coexpression of Sin with Fyn resulted in a Sin/Fyn association and a substantial increase in Sin phosphorylation in resting cells (Fig. 6D, middle panels). The Sin/Fyn association and Sin phosphorylation were reduced after two minutes of stimulation (Fig. 6D, middle panels). Similarly, Fyn immunoprecipitates from Jurkat cells stably expressing Sin revealed that more Sin associated with Fyn under resting conditions than after TCR stimulation (Fig. 6D middle panel) and that Fyn phosphorylates Sin *in vitro* (Fig. 6D, top panel). Consistent with previous results, we found no association of Sin with the Src kinase Lck in these experiments (Fig. 6D, right panels, and E). Taken together, these data suggest that Fyn associates with and phosphorylates Sin under basal conditions while TCR engagement disrupts this interaction. Our results also suggest that Fyn-mediated phosphorylation of Sin regulates the adapter function of Sin and its ability to form an inactive multi-protein complex in resting cells.

DISCUSSION

In this report we identified Sin as a negative regulator of TCR signaling as all the Sin forms inhibited TCR-induced IL-2 expression in two different cell systems.

Specifically, in transgenic T cells SinΔC expression resulted in defective proliferation and IL-2 secretion in response to TCR stimulation. Correspondingly, Sin expression in Jurkat T cells conferred defective TCR-induced transcriptional activation of IL-2-derived promoter constructs. The proliferative defect in the transgenic T cells resulted from disrupted proximal TCR signaling as the cells proliferated normally when signals downstream of the TCR were engaged. Additionally, the impaired proliferative response in SinΔC T cells was a direct consequence of inadequate IL-2 secretion as exogenous IL-2 treatment rescued the defect.

In Sin expressing Jurkat T cells the reduced activation of IL-2-promoter-derived constructs containing NFAT and AP-1 binding sites correlated with defective PLC-γ phosphorylation, as well as deficient IP3 and intracellular calcium release (Fig. 5). Activated PLC-γ cleaves the phosphoinositide PIP2 into the second messengers IP3 and DAG. IP3 production results in intracellular calcium release, which is required for NFAT activation and nuclear localization, while DAG activates PKC-θ and RasGRP resulting in increased AP-1 transcriptional activity. In T cells, NFAT and AP-1 then bind to the IL-2 promoter with other transcriptional activators to induce IL-2 expression (18, 25, 36). Thus, the inhibition of IL-2 transcription in Sin cell lines could be traced back to a block in PLC-γ activation and the calcium and transcriptional signaling pathways regulated by PLC-γ.

The exact mechanism Sin employs to block PLC-γ activation is still unclear. In Sin-expressing stable cell lines, we observed a Sin/PLC-γ interaction in resting cells and a reduction in PLC-γ phosphorylation in activated cells. The negative effect Sin has on PLC-γ phosphorylation may entirely be due to binding and sequestering PLC-γ away the

transmembrane adapter LAT. However, since we could not detect any Sin/PLC-γ association immediately after TCR stimulation, we cannot rule out that Sin may also inhibit upstream signaling events that regulate PLC-γ phosphorylation. In addition to binding LAT, PLC-γ activation requires phosphorylation by both ZAP-70 and the Itk kinases (14, 28, 41). It is conceivable that Sin binds to and interferes with Itk and Zap-70 kinase activity, as Sin contains both Itk and Zap-70 consensus binding motifs (2, and unpublished observations). Thus, Sin may regulate PLC-γ activity by direct association and sequestration, as well as yet unidentified indirect mechanisms.

Alternatively, since Sin also binds Fyn, Sin may block Fyn-mediated phosphorylation of the TCR subunits and subsequent downstream events like PLC-γ phosphorylation. Given that Jurkat T cells express approximately 30-fold more Lck than Fyn (Fig. 6D) (5), a reasonable assumption is that blocking some Fyn activity while leaving Lck activity intact would not significantly affect proximal TCR phosphorylation events. In support of this, the total TCR-induced tyrosine phosphorylation pattern in Sin overexpressing versus control Jurkat T cell lines does not appear notably different (Fig 6A, left panels). Furthermore, we could not detect alterations of CD3ζ chain phosphorylation in Sin-expressing Jurkat T cells as compared to control cells (unpublished observations). Therefore, it is unlikely that the defect in PLC-γ phosphorylation is due to a disruption in Fyn activity.

Evidence presented above reveals that the Src kinase Fyn constitutively phosphorylates Sin. This contradicts the general belief that Src kinases are inactive in resting T cells. We have previously shown that Sin can associate with Src kinases through a Src-SH3/Sin-proline interaction inducing Src kinase activity in the absence of

any extracellular stimuli. The activated Src kinase can then phosphorylate Sin Y-motifs and subsequently bind these motifs through the Src-SH2 domain (1, 38). Thus, Sin can activate Fyn under basal conditions, but considering that both the Fyn SH2 and SH3 domains can bind Sin, the affinity between the two proteins may prevent Fyn from phosphorylating other molecules. In support of this concept, Sin expression does not trigger Fyn-mediated TCR signaling events in resting cells such as CD3ζ phosphorylation and activation of TCR transcriptional targets (unpublished observations and Fig 4).

Collectively, the data presented here support a novel inhibitory role for Sin in T lymphocyte signaling that involves regulation of molecular availability and/or activity. We propose the means by which Sin negatively regulates TCR signaling is by competing with the greater-TCR complex for signaling molecules. The pattern of Sin phosphorylation, as well as its association with Fyn and PLC-γ is precisely the opposite that of the TCR complex. In uninduced T cells Sin is hyperphosphorylated and binds to Fyn, PLC-γ, and other proteins. After TCR engagement Sin becomes dephosphorylated and disassociates from Fyn and PLC-γ, which then bind to the newly phosphorylated TCR complex and LAT, respectively. The rapid rate of Sin dephosphorylation is likely due to the enzymatic activity of a phosphatase and not simply to lack of associated Fyn kinase activity. Attenuation of TCR signaling after 10 minutes evidenced as a reduction of total phosphotyrosine coincides with Sin rephosphorylation and formation of an inactive complex of TCR signaling molecules with Sin including Fyn and PLC-γ (Fig. 7). Thus, we propose that Sin acts as an adapter/scaffold protein that regulates TCR signaling by increasing the local concentration of signaling molecules for fast signal transmission

during stimulation while acting as a negative regulator by counteracting the association of molecules with the TCR in resting cells. The phosphatase responsible for Sin dephosphorylation has not yet been identified, however, it will be important to learn if the same phosphatase is responsible for subsequent TCR complex dephosphorylation and signal attenuation.

The TCR-induced dephosphorylation of Sin is intriguing given that most adapters, including positive and negative regulators, are phosphorylated after TCR stimulation (16, 26). This includes the Sin-related molecule CasL, which becomes phosphorylated upon TCR stimulation under conditions where Sin becomes dephosphorylated, i.e. overexpression in Jurkat cells. A notable exception, however, is the transmembrane protein PAG/Cbp (3, 17). In resting cells PAG/Cbp regulates Src kinases by linking them to the Src kinase-inhibitor, Csk kinase. PAG/Cbp, like Sin, binds to and is phosphorylated by Fyn in resting cells (4, 40). Upon TCR stimulation PAG/Cbp also becomes rapidly and transiently dephosphorylated, releasing Csk to the cytoplasm and allowing for Src kinase-mediated phosphorylation of the TCR subunits. The similarities between Sin and PAG/Cbp are intriguing and suggest that constitutive phosphorylation of adapter molecules by Fyn or other kinases may be a generalized mechanism for negatively regulating TCR signaling, while TCR-stimulated dephosphorylation of the adapters attenuates their opposing influence.

ACKNOWLEDGEMENTS

We thank C. Roman, A. Pernis, and S. Greenberg for critically reading the manuscript. We also thank Dr. A. Weiss for providing the Jurkat Tag cells, and the SLP-76 expression construct. This work was supported in part by the American Cancer Society Grant RPG99-09-01MGO, by the Department of Defense Grants DAMD17-99-1-9151 and DAMD17-99-1-915, by the National Institute of Allergy and Infectious Disease (NIAID) Grant RO1 AI49387-01, and the American Cancer Society's Institutional Research Grant #177F.

FIGURES

FIG. 1. Two different Sin isoforms are expressed in mature T cells. A) Schematic structure of Sin, Efs2 and SinΔC. Y = tyrosine-containing sequences, P = proline-rich motifs. B) $3x10^7$ splenocytes were immunoprecipitated with Sin-specific or isotype matched IgG control antibodies, and western blots of the immune complexes were probed with a Sin-specific monoclonal antibody to reveal expression of the Sin isoforms as indicated. C) Jurkat SV40 Tag or E6-1 cells were immunoprecipitated with Sin-specific antibody and western blots of immune complexes separated on SDS-PAGE were probed with anti-Sin antibody. Whole cell extracts (WE) from Jurkat cells overexpressing Efs2 (lane 1) or untransfected cells (lane 2) were included. Nine times more protein was loaded on lane 2 in comparison to lane 1 to reveal endogenous protein levels. D) Western blot of splenocytes from two different founders expressing SinΔC probed with anti-Sin antibody.

FIG. 2. SinΔC expression inhibits production of mature T cells. A) $1x10^6$ total splenocytes from 6-8 week old wild type and transgenic animals were triply stained with

CD4-APC/CD8-PerCP and CD3-FITC, and analyzed by flow cytometry. In the dot plots, the numbers indicate the percentage of cells in each region. Histograms represent CD3 expression within the single positive CD4 and CD8 populations in the dot plots. Two different transgenic founder lines CR1 and MA2 with their respective wild type controls are shown. B) Splenocytes were counted and stained and the percentages of total CD3 T cells and $CD4^+$ and $CD8^+$ subpopulations were determined by FACS analysis and used to calculate the actual cell numbers for each population. Results from at least 5 wild type and MA2 transgenic mice are represented as the mean $\pm$S.D.

FIG. 3. SinΔC expression inhibits T cell proliferation and IL-2 production. A) $1x10^5$ T cells purified from the spleens of 6-8 week old normal and SinΔC animals (CR1 and MA2) were stimulated with plate-bound CD3 and CD28 antibodies and PMA and ionomycin as indicated and as described in Experimental Procedures. Proliferation was calculated by [^{3}H]thymidine incorporation as the mean $\pm$S.D. radioactive count in triplicate wells. Shown is a representative of at least three experiments with two different transgenic lines (MA2 top, CR1 bottom). B) 5μl of the supernatants from stimulated cells were removed at the indicated times and analyzed by ELISA for the presence of secreted IL-2 (top graph). Purified T cells were stimulated with plate bound anti-CD3 antibody (0.05μg/ml) in the presence or absence of IL-2 (20ng/ml). Proliferation was determined by [^{3}H]thymidine incorporation (bottom graph).

FIG. 4. Sin expression inhibits TCR-induced transcriptional activation. Jurkat Tag cells were transiently transfected with increasing amounts of plasmid DNA expressing Sin (A and D), CasL (B), or SLP-76 (C) in the presence of NFAT- or AP-1-Firefly-luciferase as shown along with the HSV-TK-Renilla-luciferase reporter (A-E).

Activation fold for NFAT-and AP-1Firefly-luciferase and actual luciferase units for HSV-TK-Renilla-luciferase (A, bottom graph) are shown. E) Jurkat Tag cells were transfected with pEBB vector expressing Sin, SinΔC or Efs2 (300ng each) in the presence of the NFAT- and HSV-TK-luciferase reporters as shown. The results represent one of at least three experiments each performed in triplicate samples and fold activation is relative to the value obtained with pEBB vector backbone used to express Sin, CasL, or SPL-76 in unstimulated cells, which was given a value of 1. The results shown represent the mean ±S.D. Whole cell lysates of the transfected cells were separated on SDS-PAGE and western blotted with anti-Sin or p130Cas antibodies to reveal levels of Sin and CasL protein expression respectively (A, B, and E, insets).

FIG. 5. Defective PLC-γ phosphorylation and activation in Jurkat cells stably overexpressing Sin. A) Sin overexpressing stable Jurkat Tag cells from three different lines, were transiently transfected with NFAT-Firefly- and HSV-TK-Renilla-luciferase reporters and stimulated as described in Experimental Procedures. A representative of at least three experiments performed in triplicate samples is shown. Fold activation was determined as in Fig. 4. Sin expression levels in these cell lines are shown in the inset. B) $1x10^7$ Jurkat cells from control (vector alone) and two different Sin-expressing cell lines were stimulated with OKT3 (5μg/ml) for the indicated times and cell lysates were immunoprecipitated with anti-PLC-γ-specific antibody. Immune complexes were separated on SDS-PAGE and the western blot was probed with an anti-phosphotyrosine antibody. Total lysates of the same samples normalized for protein content were processed in parallel to reveal levels of endogenous PLC-γ. C) IP3 levels were determined as described in Experimental Procedures. One of three representative

experiments is shown. D) Intracellular calcium concentration in the presence or absence of Sin from one control and two cell lines was measured as described in Experimental Procedures. Shown is a representative of at least four experiments. E) Control or Sin-expressing Jurkat Tag ($5x10^7$) cells were stimulated with OKT3 (5μg/ml) for the indicated times and cell lysates were immunoprecipitated with PLC-γ-specific antibody. The separated immune complexes were transferred on nitrocellulose membrane, and the upper half of the membrane was probed with anti-PLC-γ (bottom panel), whereas the lower half was probed first with anti-Sin antibody (middle panel) and then stripped and reprobed with anti-phosphotyrosine-specific antibody (top panel). F) Extracts of $9x10^7$ total splenocytes from wild type mice stimulated with anti-CD3/CD28 antibodies (15μg/ml each), were immunoprecipitated with anti-PLCγ or control IgG. The upper and lower halves of the membrane containing the immune complexes were probed with anti-PLC-γ and anti-Sin antibodies respectively. Protein bands were visualized by ECL.

FIG. 6. Sin associates with and is constitutively phosphorylated by Fyn in resting cells. Sin is transiently dephosphorylated and dissociates from Fyn in response to TCR stimulation. A) $1x10^7$ Jurkat cells stably transfected with vector alone or Sin expressing plasmid were stimulated with OKT3 (5μg/ml) for the indicated time points, and whole cell extracts normalized for protein content were separated on SDS-PAGE and blotted with anti-phopshotyrosine antibody. The blot was stripped and reprobed with anti-Sin to reveal total Sin levels (lower right panel). Similarly prepared extracts from vector or Sin-expressing cell lines were immunoprecipitated with Sin-specific antibody and blotted first with anti-phosphotyrosine and stripped and reprobed with Sin antibody (right panels). B and C) Sin expressing or vector control Jurkat Tag cells (B) and $3x10^7$

thymocytes from normal mice (C), were left unstimulated or were stimulated with anti-TCR antibodies as shown. Cell extracts were immunoprecipitated with Sin-specific antibody or isotype matched control IgG and immune complexes were incubated in vitro in the presence of [^{32}P]-ATP. Immune complexes were separated, proteins transferred to nitrocellulose membrane and membranes exposed on film overnight. Subsequently, the filters were probed with Sin antibody to reveal total Sin levels. D) $5x10^6$ Jurkat Tag cells transiently transfected with Sin alone, and Sin plus Fyn or Lck were immunoprecipitated with anti-Sin antibody and processed as in A. The blots were sequentially probed with anti-phosphotyrosine, Sin, and Fyn or Lck antibodies as shown. WE = whole cell extracts. E) Control or Sin expressing Jurkat T cells were stimulated for the indicated times with OKT3 antibody and extracts were immunoprecipitated with Fyn or Lck specific antibodies. Immune complexes were subjected to in vitro kinase assays in the presence of radioactive ATP and subsequently separated on SDS-PAGE. Blots were exposed on film and then probed sequentially with Sin, and Fyn, or Lck antibodies as shown.

FIG. 7. Model for Sin mediated regulation of TCR signaling. In resting T cells Sin is constitutively phosphorylated by Fyn and bound to PLC-γ and other proteins (1). TCR stimulation induces the dephosphorylation of Sin through the action of a tyrosine phosphatase, which correlates with the rapid and transient release of associated substrates including Fyn and PLC-γ (2). Fyn and PLC-γ bind to their respective targets to mediate TCR signaling. Attenuation of TCR signaling 10 minutes after stimulation results in reassociation of Fyn and PLC-γ with Sin and Sin phosphorylation, reformation of the inactive complex, and return to resting conditions (1).

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Fig. 1

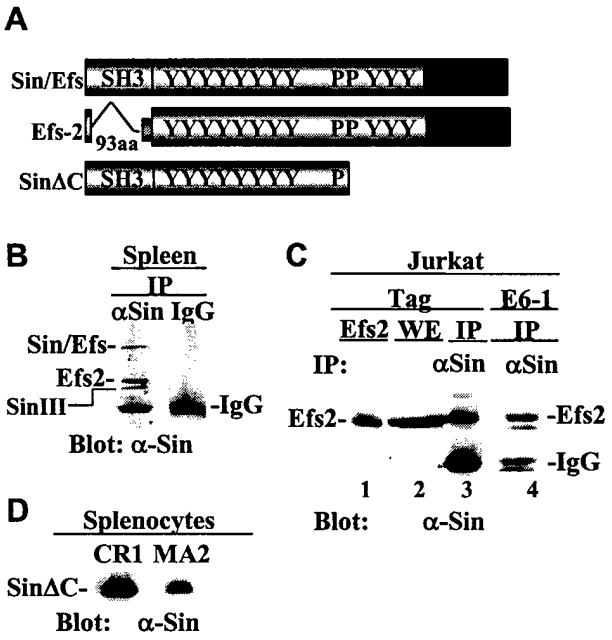
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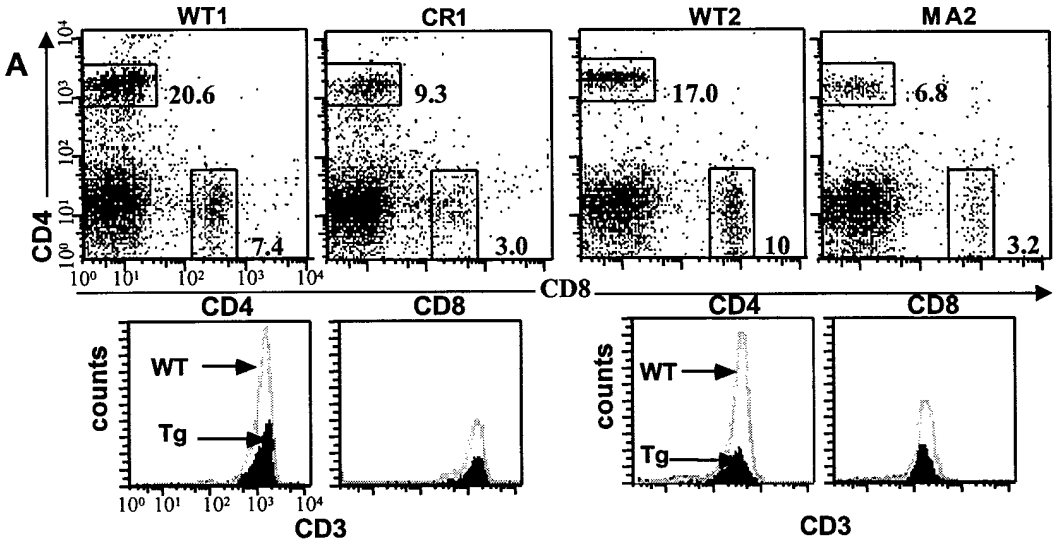
Fig. 2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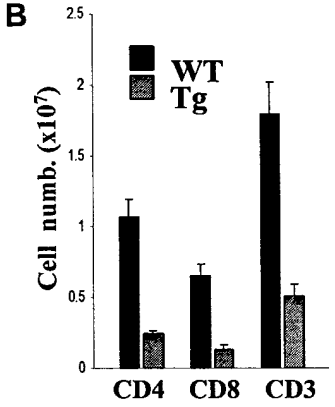
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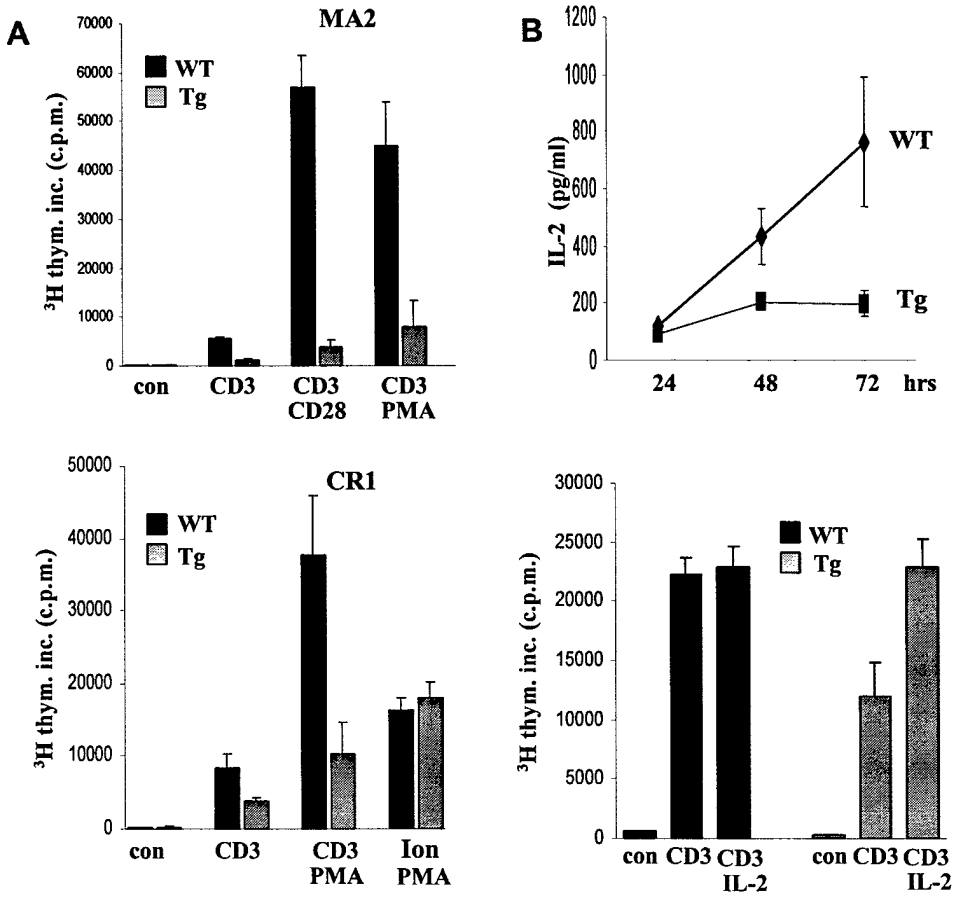
Fig. 3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con
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CD3 CD28
CD3 PMA
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IL-2 (pg/ml)
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1000
800
600
400
200
0
WT
Tg
24
48
72
hrs
CR1
WT
Tg
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40000
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20000
10000
0
con
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Ion PMA
^{3}H thym. inc. (c.p.m.)
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10000
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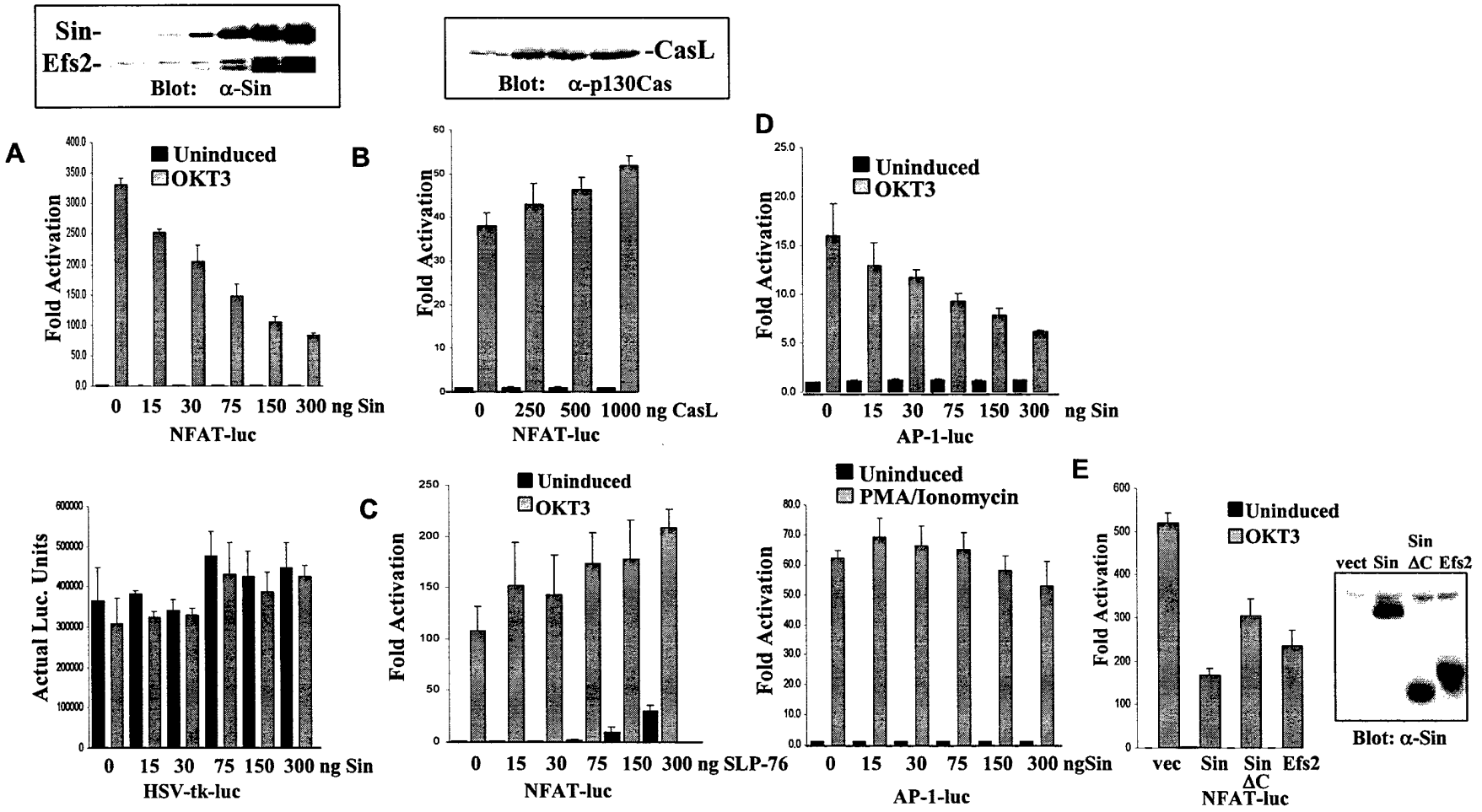
CD3
CD3 IL-2
con
CD3
CD3 IL-2

Fig. 4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Efs2-
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Blot: α-p130Cas
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NFAT-luc
B
Fold Activation
0 250 500 1000 ng CasL
NFAT-luc
D
Uninduced
OKT3
Fold Activation
0 15 30 75 150 300 ng Sin
AP-1-luc
Actual Luc. Units
0 15 30 75 150 300 ng Sin
HSV-tk-luc
C
Uninduced
OKT3
Fold Activation
0 15 30 75 150 300 ng SLP-76
NFAT-luc
Uninduced
PMA/Ionomycin
Fold Activation
0 15 30 75 150 300 ngSin
AP-1-luc
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NFAT-luc
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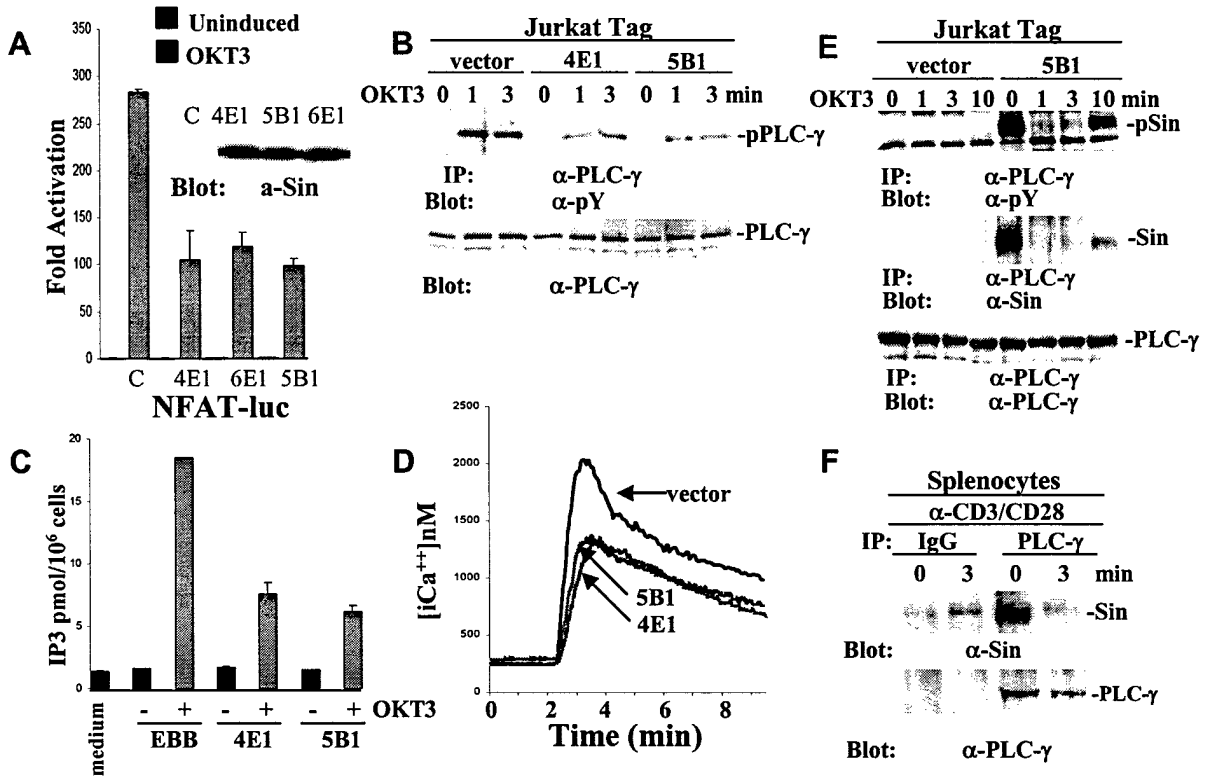
Fig. 5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Jurkat Tag
vector 4E1 5B1
OKT3 0 1 3 0 1 3 0 1 3 min
-pPLC-γ
IP: α-PLC-γ
Blot: α-pY
-PLC-γ
Blot: α-PLC-γ
E
Jurkat Tag
vector 5B1
OKT3 0 1 3 10 0 1 3 10 min
-pSin
IP: α-PLC-γ
Blot: α-pY
-Sin
IP: α-PLC-γ
Blot: α-Sin
-PLC-γ
IP: α-PLC-γ
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C
IP3 pmol/10^6 cells
medium
- + - + - + OKT3
EBB 4E1 5B1
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4E1
Time (min)
F
Splenocytes
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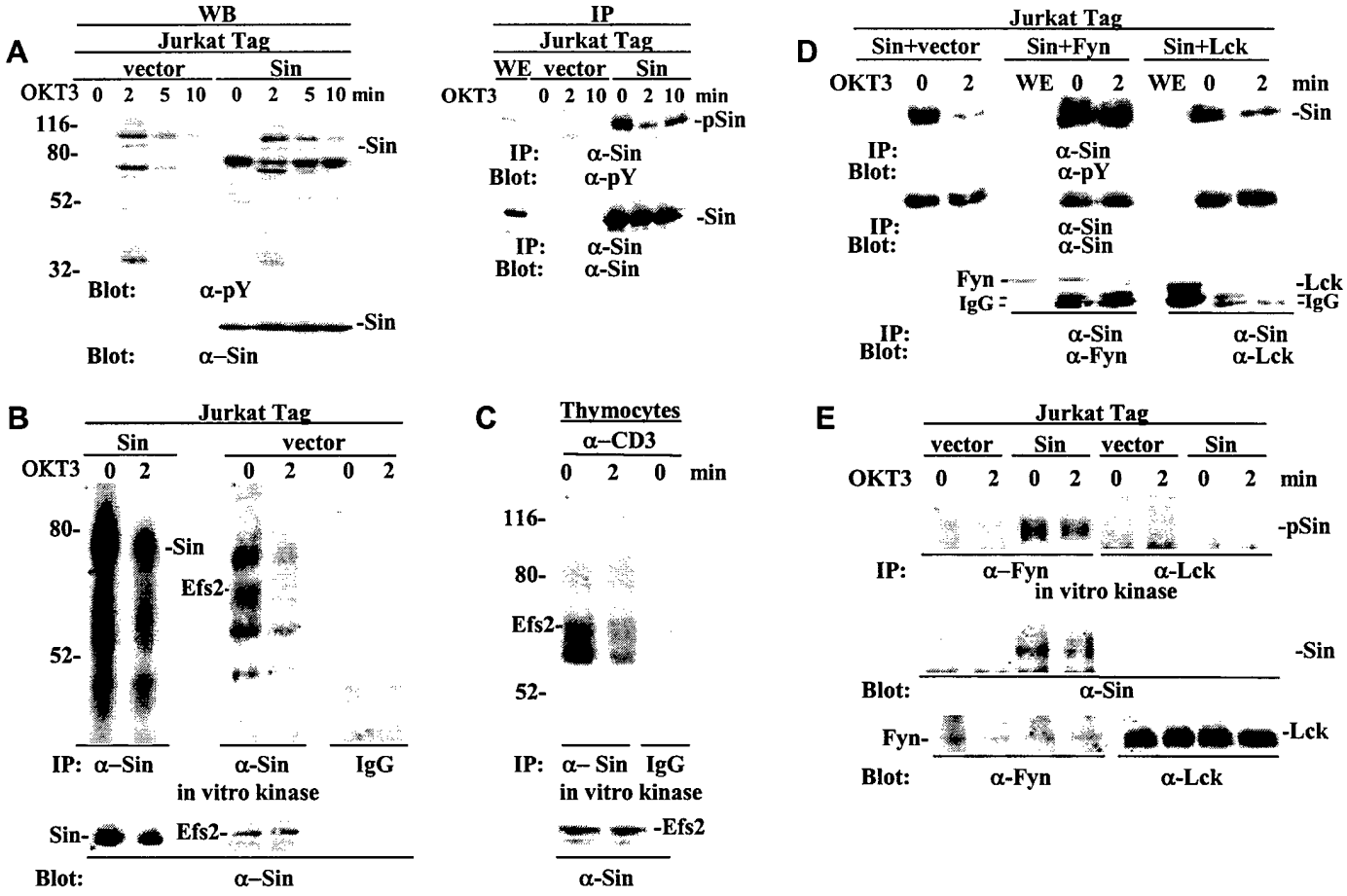
IP: IgG PLC-γ
0 3 0 3 min
-Sin
Blot: α-Sin
-PLC-γ
Blot: α-PLC-γ

Fig. 6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vector
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OKT3 0 2 5 10 0 2 5 10 min
116-
80-
52-
32-
-Sin
Blot: α-pY
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WE vector Sin
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-Sin
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in vitro kinase
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Thymocytes
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Efs2-
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OKT3 0 2 0 2 0 2 0 2 min
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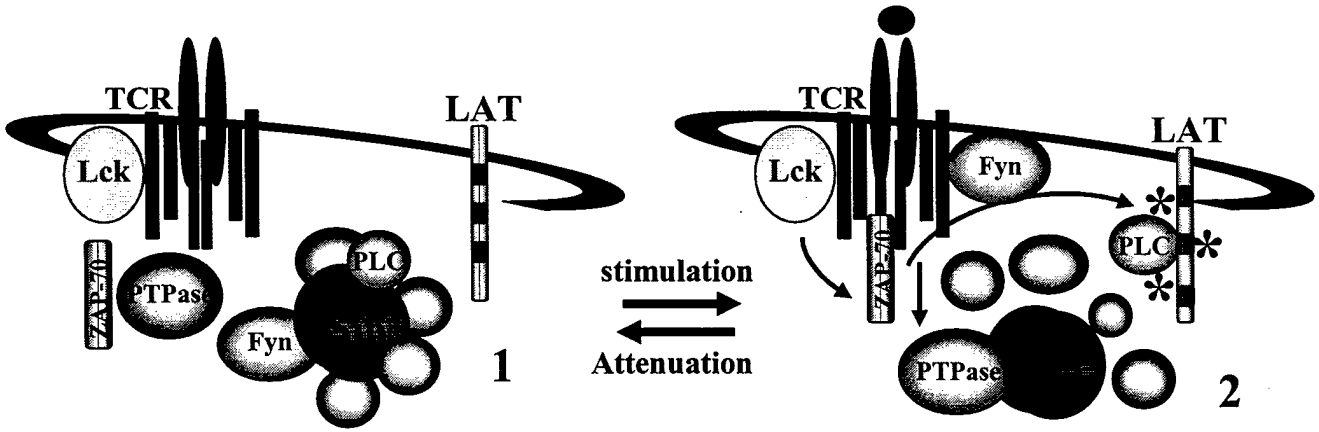
-Lck
Blot: α-Fyn α-Lck

Fig. 7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PLC
PTPase
2

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Sin: good or bad? A T lymphocyte perspective

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Acknowledgements
This work was supported in part by the American Cancer Society Grant RPG99-09-01MGO, by the Department of Defense Grants DAMD17-99-1-9151 and DAMD17-99-1-915, by the National Institute of Allergy and Infectious Disease (NIAID) Grant RO1 AI49387-01, and the American Cancer Society's Institutional Research Grant #177F and the Herbert Irving Comprehensive Cancer Center. L.D. and was supported by NIH grant 5 T32 AI07525 and A.R. by NIH grant 5 T32 GM03767-26. L.X. was supported in part by the American Cancer Society Grant RPG99-09-01MGO. K.A. was supported in part by the Department of Defense Grants DAMD17-99-1-9151 and DAMD17-99-1-915, the National Institute of Allergy and Infectious Disease (NIAID) Grant RO1 AI49387-01, and the American Cancer Society Grant RPG99-09-01MGO.

Immunological Reviews 2003
Vol. 192: 181–195

Immunological Reviews
0105-2896

Summary: Stimulation of T cells through their antigen receptor induces a multitude of signaling networks that regulate T cell activation in the form of cytokine production and T cell proliferation. Multiple signal integration sites exist along these pathways in the form of multiprotein signaling complexes, the formation of which is facilitated by adapter and scaffold molecules. In recent years a number of adapter and scaffold molecules have been described in T cells and shown to play an integral part in T cell function. Among these molecules are proteins that function as positive or negative regulators of T cell activation downstream of the activated T cell receptor (TCR). Here, we discuss the role of a small family of multi-adapter proteins on T cell activation, the p130Cas family, with emphasis on one of its members, Sin (Src-interacting protein). Our results suggest that Sin inhibits thymocyte development and T cell activation and is a novel negative regulator of T lymphocyte function.

Introduction

Adapter proteins have no known enzymatic or transcriptional activity but participate in signaling events by mediating protein –protein interactions that lead to the formation of multiprotein complexes or signalosomes (1). The formation of signalosomes is mediated by reciprocal interactions between conserved domains and their binding motifs present on the adapter molecules and their interacting partners. The most widely expressed conserved domains found on adapters include the Src homology regions 2 and 3 (SH2 and SH3, approximately 100 and 50 amino acids long, respectively) (2), the phosphotyrosine-binding (PTB) domain (3,4), the tryptophan-tryptophan (WW) (5), pleckstrin homology (PH) (6), and postsynaptic density-disk large-zo1 (PDZ) domains (4). SH2 and PTB domains bind to phosphorylated tyrosine residues on target molecules (7,8); SH3 and WW domains recognize and bind to proline-rich sequences (5,9,10). PH domains bind to phospholipids (11), and PDZ domains bind short sequences that contain conserved, hydrophobic residues in their C-terminus (4,12). In addition to these conserved domains, most adapter molecules themselves contain proline- and tyrosine-based motifs that bind to respective

SH3 and SH2 modular domains and promote complex reciprocal interactions between adapters and their cellular partners.

Molecules that mediate protein–protein interactions can be classified as adapter or scaffold proteins depending on their combination of conserved modular domains and/or proline/tyrosine-based motifs. *Bona fide* adapters are molecules that consist predominantly of modular domains serving to bridge two signaling molecules, whereas scaffold proteins contain modular domains as well as tyrosine and proline motifs and bind to two or more proteins simultaneously (13). T cells express both adapter and scaffold molecules, and several excellent reviews have been published recently discussing the function of these molecules in T cell receptor (TCR) signaling (14–19). Therefore, we discuss briefly only those molecules whose function has been supported by gene targeted experiments in mice. For the most part, we focus our attention on the p130Cas family of scaffold molecules, a small protein family consisting of three members, with emphasis on the least characterized member, Sin. We start our discussion with a description of the structural characteristics shared by the three proteins. Then we give an overview of the literature pertaining to the function of p130Cas and Cas-L in T lymphocytes, and proceed with a more detailed discussion on the role of Sin in T lymphocytes based on evidence generated in our laboratory.

Adapters and scaffolds in T cells

T cells express a variety of both adapter and scaffold molecules. Adapters include the Grb2 and Gads proteins, which link linker for activation of T cells (LAT) to the son of sevenless (Sos) nucleotide exchange factor and SLP-76, respectively (20–23). Both adapters consist mainly of SH2 and SH3 domains, one N- and one C-terminal SH3 domain flanking a central SH2 domain. The importance of these molecules in T lymphocyte function has been confirmed by targeted disruption of the genetic loci encoding for these adapters. Thus, targeted disruption of the Gads locus leads to defective thymocyte development and uncouples the association of SLP-76 and LAT (24), whereas haploid insufficiency of Grb2 inhibits negative selection of thymocytes and reduces TCR-induced activation of the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinases (25). The Src-like adapter protein (SLAP) is yet another recently identified molecule that consists of one SH2 and one SH3 domain, shares structural homology with Lck and is a negative regulator of T lymphocytes (26, 27). Like Lck, SLAP consists of an N-terminal SH3 followed by an SH2 domain (these are 50% and 51% homologous to those of Lck), as well as a unique C-terminal tail of unknown function. Unlike Lck, SLAP has no kinase domain and is therefore classified as an adapter. SLAP expression is restricted to lymphoid tissues (28), and mice deficient in SLAP exhibit marked upregulation of TCR and CD5 expression at the double positive (DP, $CD4^+CD8^+$) stage, as well as increased positive selection (29). Thus, SLAP is thought to regulate T lymphocyte function by mediating TCR downregulation at the DP stage and thus regulating positive selection of thymocytes (29).

A number of scaffold molecules are also expressed in T lymphocytes that positively or negatively regulate T lymphocyte responses. The best-characterized positive regulators of T lymphocytes include LAT, SLP-76, and SLAP-130/FYB (14–19). SLP-76 and LAT are scaffold molecules that mediate the formation of a signaling complex that stimulates intracellular calcium release through the activation of phospholipase C (PLC-γ). The important roles of these proteins in T lymphocyte function have been substantiated with gene targeting experiments showing that SLP-76 and LAT are required for normal thymocyte development (30–32). Reconstitution experiments, in which mutant forms of SLP-76 and LAT were introduced into the SLP-76 and LAT null backgrounds, showed that these proteins are also important in mature T cell signaling. These experiments also revealed differential requirements for different conserved domains in T cell development and activation (31, 33, 34). A third scaffold protein ADAP/SLAP-130/Fyb has been recently shown to link the TCR to the actin cytoskeleton through LAT and SLP-76 and to bind to proteins such as Nck, Wiskott–Aldrich syndrome protein (WASP), Vasp and Arp2/3 involved in actin polymerization (35). ADAP/SLAP-130/FYB knockout mice exhibit reduced T cell proliferation and cytokine production, as well as impaired TCR-induced integrin clustering and adhesion (36, 37).

Scaffold proteins involved in the negative regulation of TCR signaling include the Cbl family of proteins (17, 38, 39), and a recently characterized transmembrane regulator of TCR signaling PAG/Cbp (protein associated with glycosphingolipid-enriched microdomains/Csk-binding protein), among others (40, 41). The function of Cbl in T lymphocytes has been studied extensively and represents the prototype of negative regulators of T cells. This protein associates with a variety of signaling molecules such as Src kinases, Crk, Grb2, PI3K and ZAP-70, and regulates TCR signaling through different mechanisms (39). These mechanisms include downregulation of enzymatic activity of tyrosine kinases, interference with normal Ras activation by increasing Rap1 GTP levels and

sequestering Raf away from Ras (42), or degradation of proteins through E3 ligase-mediated ubiquitination (43–45). Two types of Cbl-deficient animals have been described, Cbl-b$^{-/-}$ and c-Cbl. Cbl-b$^{-/-}$ mice show normal thymocyte development but develop spontaneous autoimmunity due to reduced T cell activation thresholds (46,47). c-Cbl-deficient thymocytes, on the other hand, exhibit increased expression of CD3 and CD4 molecules, display increased positive selection and markedly upregulated activity of ZAP-70 and the MAP kinase ERK (48, 49). Collectively, these observations implicate these molecules as important negative regulators of T lymphocyte function.

PAG/Cbp is a transmembrane protein that localizes to glycosphingolipid-enriched microdomains of the plasma membrane (40,41). PAG/Cbp is thought to regulate the activity of Src kinases by linking them to Csk kinase, a known Src inhibitor that induces the inactive conformation of Src kinases. PAG/Cbp is constitutively phosphorylated in unstimulated cells and bound to the Csk kinase through Cbp-tyrosine motifs/Csk-SH2 domain interactions. Upon TCR stimulation, PAG/Cbp becomes dephosphorylated rapidly and releases Csk to the cytoplasm, which allows Src kinases to become activated and initiate TCR signaling. Thus, PAG/Cbp appears to regulate T cell activation by controlling the activity of Src kinases through reversible recruitment of Csk (14,15). PAG/Cbp-deficient mice have not yet been reported.

Characteristics of the Cas family of proteins

The Cas family of proteins is a relatively recently identified group of proteins consisting of three members: p130Cas, HEF1/CasL and Sin/Efs. p130Cas (Crk-associated substrate) was the first member of the family to be identified. It is ubiquitously expressed, and substantial evidence suggests a role for this protein in integrin receptor signaling and in the processes of cell adhesion and motility (50,51). The second member, HEF1 (human enhancer of filamentation), was isolated in a screen for human proteins that conferred morphological changes leading to budding in yeast (52) and also as CasL (Crk-associated substrate in lymphocytes), a protein involved in T cell receptor and β-1 integrin-mediated signaling in T lymphocytes (53,54). HEF1 expression is highest in epithelial cells and T and B lymphocytes. Sin (Src-interacting protein), cloned by us and independently by Ishino et al. as Efs (embryonal Fyn substrate), is a substrate for Src kinases and is most highly expressed in the embryo, brain and thymus (55–57). Evidence generated in our laboratory suggests that Sin plays a role in TCR-mediated T lymphocyte development and activation (58). In our subsequent discussion we refer to Sin/Efs as Sin, and the terms HEF1 and CasL are used interchangeably as pertaining to specific studies.

The Cas proteins are defined as multiadapter/scaffold/docking molecules that lack any known enzymatic activity but instead mediate signaling by promoting protein–protein interactions through conserved sequence motifs (51,54,59). These conserved motifs consist of the following: (a) SH3 domains that are highly conserved (80–90%) among the three family members and have the potential to bind to proline-rich motif-containing proteins; (b) a central substrate-binding region containing tyrosine residues embedded within specific conserved sequences that determine the binding specificity of these tyrosines for different SH2 domains (7). Once phosphorylated by Src or other kinases, these tyrosine residues bind to the SH2 domains of defined cytoplasmic intermediates, thus forming unique multiprotein signaling complexes; (c) proline-rich sequences with binding specificity for Src kinase SH3 domains; and (d) a conserved C-terminus (~60%) of approximately 135 amino acids of unknown function (*Fig.* 1).

In agreement with the originally predicted function of the Cas proteins as docking proteins, a plethora of *in vitro* and *in vivo* interacting partners have been identified to date (reviewed in 51). For example, the N-terminal SH3 domains of the Cas proteins have been shown to interact with proline-rich motifs found on the focal adhesion kinase (FAK) and the related protein Pyk2/RAFTK/Cakβ. These proteins are tyrosine kinases involved in integrin receptor signaling and were shown to interact with the SH3 domains of p130Cas and HEF1 *in vivo* and with all three Cas proteins *in vitro*. In addition, the phosphatases PTP-1B and PTB-PEST have been shown to interact with the SH3 domains of Cas, HEF1 and Sin *in vitro* (51).

The central, substrate-binding regions of the Cas proteins contain multiple tyrosine-based motifs that, when phosphorylated by Src and other kinases, can bind to SH2-domain-containing signaling intermediates (*Tables* 1 *and* 2). In addition, Cas and Sin contain one and two proline-rich motifs, respectively, that exhibit high binding specificity for Src kinase SH3 domains (*Table* 3). The overall conserved motifs of the Cas proteins in these regions suggest that these molecules may have overlapping functions. However, the types and numbers of the conserved motifs differ and together with the varied length and amino acid composition of the Cas proteins argue for unique functional roles for these molecules. Indeed, a motif-based profiling of potential binding partners for the Cas proteins using a peptide-based searching algorithm (60)

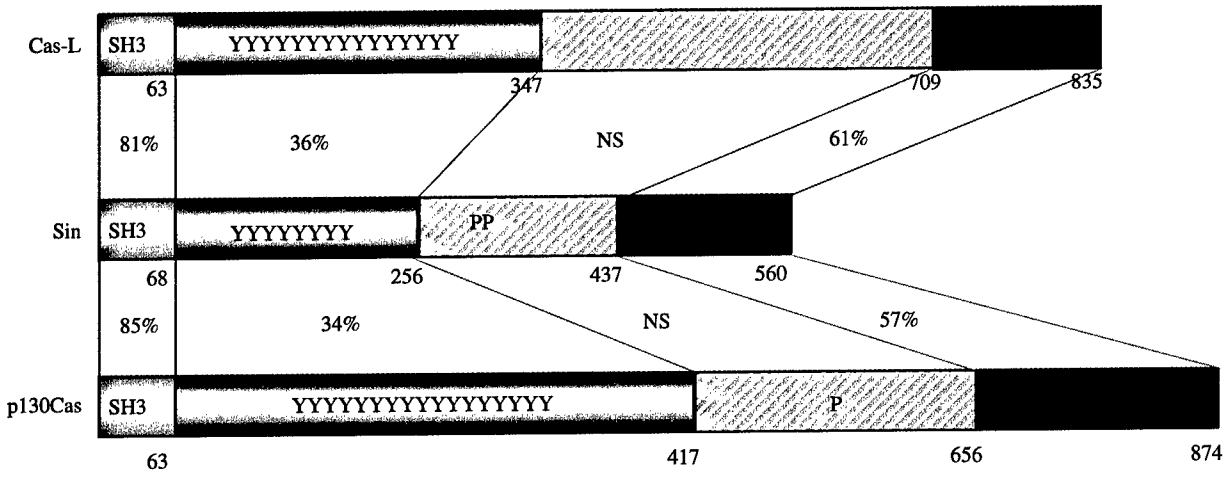

***Fig. 1.* Schematic structure representation and homology comparison of the Cas protein family.** Amino acid homologies within the SH3, substrate and C-terminal domains are as shown. Homologies were determined by pairwise sequence alignment between Sin and CasL and Sin and p130Cas. Numbers below each domain correspond to the last amino acid residue in the domain. NS represents no significant homology. Y represents the YXXP motifs on each protein, while P represents the proline-rich motifs that bind to Src kinase SH3 domains.

revealed common as well as unique binding partners, supporting both overlapping and divergent functional roles for these proteins (*Table 1*). The search results show that all Cas proteins contain tyrosine motifs that are potential phosphorylation sites for the Abl kinase and binding sites for the Crk, Nck and Itk SH2 domains. In addition, Sin and Cas (but not CasL) can bind to the SH2 and SH3 domains of PLC-γ as well as to Src kinase SH3 domains (*Table 3*). Remarkably, although Sin has the least number of tyrosine-based motifs, it exhibits the highest degree of divergence in the number and type of potential binding partners (*Table 1*). Whether this finding has physiological significance or contributes to functional divergence as compared to Cas and CasL remains to be determined.

Table 1. Predicted binding partners of Cas family members

Binding partners	Binding sites on: Sin	CasL	p130Cas
Kinase domains			
Abl-kinase	2	4	9
Akt-kinase	1	–	–
Calmodulin-dependent kinase	–	1	–
Lck-kinase	–	–	1
DNA-PK	–	1	–
ERK-1	1	–	–
GSK3 kinase	2	–	–
PKC-ε	–	1	–
SH2 domains			
Abl-SH2	–	1	6
Crk-SH2	4	10	12
Grb2-SH2	–	1	–
Itk-SH2	1	2	3
Nck-SH2	3	6	8
PLC-γ SH2	1	–	2
Src-SH2	1	1	1
SH3 domains			
Cortactin-SH3	1	–	–
Grb2-SH3	2	–	–
Crk-SK3	–	–	1
Nck-SH3	1	–	–
PLC-γ SH3	1	–	1
Src, Fyn-SH3	2	–	1

The predicted binding partners of Cas family members were determined using a peptide-based algorithm (60) accessible through the website http://scansite.mit.edu. Domains within proteins that bind to all Cas proteins are shown in red, domains that interact with two out of three Cas family members are shown in green.

Some of the predicted interactions between the tyrosine motifs of the Cas proteins and proteins listed in *Table 1* have been previously shown to occur *in vitro* and *in vivo*. Thus, Sin, Cas and HEF1 interact with Crk *in vivo* (61–64), and HEF1 interacts with Abl, Nck and Lck *in vitro* (53, 65, 66). We have also shown that the proline-rich motifs of Sin interact with Src kinase SH3 domains *in vitro* (67); Sin and Src interact *in vivo* (61). Fyn also interacts with Sin (Efs) *in vivo* (56). While most of these associations have been studied in cell systems other than T lymphocytes, the interactions of the Cas proteins with Src kinases, Crk, Itk, Nck and PLC-γ are also likely to be important in T cell signaling, given that the involvement of these molecules in T lymphocyte activation is well established. The association of the Cas proteins with other molecules and their physiological significance are discussed further in the following sections.

As mentioned above, the Cas family members have a conserved C-terminus consisting of approximately 135 amino acids of unknown function. It has previously been shown that the C-terminal domain of HEF-1 contains a divergent helix–loop–helix motif that can homodimerize or promote

Table 2. Binding partners for Sin, CasL and p130Cas tyrosine-based motifs

Sin		CasL			p130Cas	
EQEV Y-VIP	Crk, Itk, PLC-γ SH2	ARAL Y-DNV		– MK Y-LNV		
SDSI Y-KVP	Crk SH2	QQKL Y-QVP		AKAL Y-DNV		
VAEV Y-DVP	Crk Nck SH2	RDTI Y-QVP	Crk SH2	LVGM Y-DKK		
SSCP Y-DSP	Nck SH2	VPPS Y-QNQ	Grb2 SH2	PASQ Y-SPM		
DEAP Y-DVP		NQGI Y-QVP	Abl kinase	LPTA Y-QPQ		
GPPL Y-AAP	Abl kinase	NQDV Y-QVP	Crk SH2	SDNV Y-LVP	Abl kinase, Crk, PLC-γ SH2	
LLNL Y-EAP	Abl kinase	TGLG Y-VYE		QQGL Y-QAP	Abl kinase	
DEGI Y-DVP	Crk, Nck SH2	LGYV Y-EYP		ATDL Y-QVP	Abl kinase, SH2 Crk, Nck, Itk SH2	
CLPG Y-GGL		YVYE Y-PSR		AQDI Y-QVP	Abl kinase, SH2 Crk SH2	
PHNE Y-EGI		YPSR Y-QKD		GHDI Y-QVP	Crk SH2	
MAEEY-DYV	Src, Fyn SH2	QKDV Y-DVP	Crk, Nck SH2	VGQG Y-VYE		
EEYD Y-VHL		TQGV Y-DIP	Crk, Itk, Nck SH2	QMYV Y-EAS		
LLHF Y-AGQ		PQGV Y-DIP	Crk, Nck SH2	EQDE Y-DTP	Nck SH2	
QSHY Y-SAL		TQGV Y-AIP	Abl kinase, Itk SH2	PQDI Y-DVP	Abl kinase, SH2 Crk, Nck SH2	
AALG Y-PSD		REKE Y-DFP	Crk, Nck SH2	LPNQ Y-GQE		
		PEGV Y-DIP	Abl kinase, Crk, Nck SH2	GQEV Y-DTP	Crk, Nck SH2	
		QSDA Y-DVP	Crk SH2	LLDV Y-DVP	Crk, Nck SH2	
		RDGV Y-DVP	Abl kinase, Crk, Nck SH2	HHSV Y-DVP	Abl, Crk, Nck SH2	
		IEKL Y-RLQ		REET Y-DVP	Crk, Nck, Itk SH2	
		DWRC Y-GYM	Crk, Nck SH2	AEDV Y-DVP	Abl kinase, SH2	
		RCYG Y-MER		APDL Y-DVP	Abl kinase, SH2 Crk, Nck SH2	
		FLRE Y-LHF		PGTL Y-DVP	Abl kinase	
		TIST Y-AET		DDGV Y-AVP	Abl kinase, Crk, Itk, PLC SH2	
		NSSE Y-THP		MEDV Y-QTL	Lck kinase	
		HPGD Y-KAQ		PDGQ Y-ENS		
		WMDDY-DYV	Src, Fyn SH2	**WMEDY-DYV**	Src, Fyn SH2	
		DDYD Y-VHL		EDYD Y-VHL		
		LLCF Y-YDQ		LLLF Y-LEQ		
		LCF Y-DQC		SVTH Y-SNL		
		AALH Y-PST		AALQ Y-PSP		

The tyrosine-based motifs of the Cas proteins and potential binding SH2 domains were determined using the scansite algorithm as described in *Table 1*. Phosphaylated tyrosine residues are shown in red YXXP and YDXP motifs that bind to Crk and Nck SH2 domains, respectively, are shown in green. The YDYV (bold) motif is present in Cas proteins and binds to Src kinase SH2 domains.

heterodimerization with other proteins (68). Recently, it has also been shown that the conserved C-terminal domain of p130Cas binds to Chat, a novel guanine nucleotide exchange factor (GEF) with specificity for the Rap1 and R-Ras GTPases. Two Chat isoforms have been described thus far, one that is expressed ubiquitously (Chat) and one that is expressed only in hematopoietic cells, the thymus, lymph node and spleen (Chat-H) (69). We have recently obtained evidence that Sin also binds to Chat and Chat-H and that this interaction requires the conserved C-terminus of Sin (unpublished data). The

Table 3. Binding partners for Sin and p130Cas proline-rich motifs

Sin		CasL	p130Cas	
QDRPL-PPPP	Src, Nck, Grb2, SH3 Cortactin,	–	TPAQ-PLVP	Crk SH3
PPQHR-PRLP	PLC-γ SH3	–	QSRPL-PSPP	Src SH3
SRRPL-PALP	Src, Fyn, Lyn Hck, SH3	–		

The proline-rich motifs of Sin and p130Cas and potential interacting SH3 domain partners are shown and were determined using the scansite algorithm as described in *Table 1*. The core PXXP, SH3 binding motifs are shown in red.

physiological significance of these interactions is under investigation.

P130Cas and HEF1/CasL function in nonlymphoid cells

p130Cas was isolated as a 130-kDa protein that was highly phosphorylated in v-Crk and v-Src transformed cells and it was named Cas based on its ability to associate with Crk (Crk-associated substrate) (50). HEF1/CasL is a 105-kDa protein that was isolated as HEF1 in a screen for human proteins that confer morphological changes in yeast leading to hyphae projections and filamentous budding (52). It was also identified as CasL, a previously uncharacterized, p130Cas-related protein that was phosphorylated in response to integrin receptor engagement in T-lymphoblastoid H9 and peripheral T cells (53, 54). Existing evidence supports a role for Cas and HEF1 in integrin signaling and the integrin-dependent processes of cell adhesion and migration (51). Studies in nonlymphoid cells have shown that p130Cas and HEF1 localize mainly at focal adhesions and stress fibers, although the proteins also exhibit some cytosolic distribution. Phosphorylation of the proteins correlates with the presence of the proteins in membranous and insoluble cytoskeletal fractions, whereas nonphosphorylated proteins localize mainly to the soluble cytosolic fraction. Localization of the Cas proteins to the focal adhesions is mediated by interaction of the Cas family SH3 domain with proline-rich regions on FAK, a kinase that has been implicated in integrin receptor signaling (70). Upon integrin receptor stimulation, FAK becomes autophosphorylated creating a binding site for Src-family kinases. Cas proteins then become phosphorylated by Src kinases or by the concerted action of FAK and Src kinases, leading to the recruitment of other proteins and the formation of a multiprotein signaling complex that transduces integrin-dependent signals to the nucleus (51, 71, 72).

Given that integrin receptors regulate the ability of cells to form and disrupt extracellular attachments, the presence of the Cas proteins in focal adhesions and their interactions with FAK and other molecules suggest a physiological role for these proteins in cell adhesion and motility. This suggested role is supported by gene knockout experiments that show that p130Cas- and FAK-deficient fibroblasts exhibit reduced motility, which correlates with increased numbers of focal adhesions and, as a consequence, increased cell adhesion (73).

Although the role of p130Cas in cell adhesion and migration has been extensively studied in nonlymphoid cells, limited evidence exists about the role of this protein in T lymphocytes. Recently, p130Cas was implicated in T cell chemotaxis in response to SDF-1α chemokine stimulation of human Jurkat T cells. In this cell system, it was shown that WASP, p130Cas, Nck and FAK are all phosphorylated in response to Jurkat T cell stimulation with SDF-1α, and that Nck, Cas and FAK associate with one another in immunoprecipitation assays (74). Together these data support a role for p130Cas in cell migration and suggest that Cas may be required for T cell chemotaxis.

Role of CasL in T lymphocytes

CasL, a protein predominantly expressed in cells of lymphoid lineage, was identified as a 105-kDa protein in H9 human T cells and is inducibly phosphorylated in response to integrin receptor stimulation (53). Cloning of the CasL cDNA revealed a novel protein with structural homology to p130Cas. Initial studies showed that integrin-induced CasL phosphorylation leads to *in vivo* interaction of CasL with cellular proteins such as FAK, the adapters Crk and Nck, and the tyrosine phosphatase SHP2 (53). As described earlier for p130Cas, phosphorylation of CasL is triggered through integrin-mediated activation of FAK. FAK subsequently phosphorylates CasL as a result of interactions between the CasL-SH3 domain and proline-rich motifs on FAK (75). FAK-mediated phosphorylation of CasL then leads to the recruitment of the Src kinase Fyn, through interactions of the Fyn-SH2 domains with the YDYVHL tyrosine-containing motif of CasL (*Table* 2). Fyn either independently or together with FAK then further phosphorylates CasL on tyrosine residues (75).

CasL has also been demonstrated to play a role in antigen receptor signal transduction in response to TCR crosslinking in human H9 and peripheral T cells (65, 76). As with integrin stimulation, TCR crosslinking leads to transient CasL phosphorylation and CasL association with the Crk/C3G protein complex *in vivo* (65, 76). In contrast to integrin ligation, FAK activation and phosphorylation is not required for TCR-induced CasL phosphorylation, because a CasL-SH3 domain deletion mutant that does not bind to FAK is still phosphorylated in response to TCR stimulation (76). Consistent with this observation, FAK fails to become phosphorylated in response to TCR crosslinking. Instead, TCR-dependent phosphorylation of CasL is mediated by the Src kinases Fyn and Lck. This phosphorylation is specific, as the related kinase ZAP-70, which is also important in TCR signaling, does not associate with or phosphorylate CasL in the same experiments (77).

CasL is also phosphorylated when antibodies against integrin receptors and the TCR are used for costimulation of T cells (78). Thus, under these conditions it was found that CasL is

required for interleukin-2 (IL-2) production upon costimulation of TCR/integrin receptors, and that the SH3 domain of CasL is necessary for integrin receptor- but not TCR-mediated stimulation. Using human Jurkat T cells, which have impaired integrin signaling, the authors showed that overexpression of full-length CasL is able to restore integrin/TCR-induced IL-2 production, whereas an SH3 deletion mutant of CasL was unable to do so (78). This inability is due to the absence of CasL-SH3/FAK proline motif interaction, which is required for integrin receptor-mediated Cas phosphorylation (76). These results suggest that CasL is involved in integrin and TCR receptor signaling and that CasL is a positive regulator of T cell function. Definitive evidence for the role of CasL in T lymphocyte function awaits the generation of CasL-deficient animals.

Cloning of Sin/Efs

We isolated Sin as an Src-interacting protein by screening a 16-day mouse embryonic expression library using the biotinylated SH3 domains of Src kinases as probes (67). In the initial screen, a partial cDNA clone was isolated that contained two 7-amino-acid-long sequences with core proline-rich motifs (PXXP) that bound with high affinity to the Src, Fyn, Hck and Lyn SH3 domains *in vitro* (*Table* 3) (67). The binding specificity of the Sin proline-rich motifs was restricted to Src kinase SH3 domains and did not recognize the SH3 domain of the related Abl protein tyrosine kinase, suggesting that Sin was a specific Src kinase ligand. Isolation of a full-length cDNA clone revealed that Sin displayed structural and primary sequence homology to p130Cas and HEF1 and thus was the third member of this family of proteins to be cloned (55). Sin was also independently cloned as Efs from the same embryonic expression library using biotinylated Fyn-SH3 domain as a probe (56). The expression patterns of Sin/Efs were confirmed by Northern blots, which demonstrated that Sin is indeed expressed in the mouse embryo and that in adult tissues Sin is highly expressed in the brain, brainstem, thymus and skeletal muscle (56) (*Fig.* 2B,C). Subsequent analysis by reverse transcriptase polymerase chain reaction (RT-PCR) revealed more widespread expression of Sin/Efs (57).

At least two different Sin mRNAs can be detected on Northern blots with RNA from brain and thymus (*Fig.* 2B) (57). Consistent with this two protein isoforms of Sin have been cloned thus far, full-length Sin and Efs2, a truncated Sin/Efs isoform that is missing the conserved N-terminal SH3 domain (55–57). Efs2 was isolated from a normal, 2-year-old human female hippocampus (57). Thymocytes and T cells from adult mice express at least three isoforms that are recognized by a Sin-specific antibody whereas the adult mouse brain expresses only full-length Sin (*Fig.* 2C) (58). In addition, we have isolated full-length and Efs2 cDNA clones from a mouse thymus library as well as a third partial cDNA clone (Sin-III) that contains an internal deletion that removes the proline-rich motifs of Sin that mediate interactions with Src kinase SH3 domains (*Fig.* 2A). We believe that the third protein band that appears in Sin immunoprecipitates is Sin-III, although definitive proof requires the isolation of full-length cDNA clone. The functional significance of these different isoforms is not clear at present, and is under investigation. However, given the data mentioned above that an SH3 deletion mutant of CasL is involved in T cell but not integrin receptor signaling, it is possible that these different isoforms are differentially involved in signaling through these receptors. In our experiments thus far, we have not examined the involvement of Sin in integrin receptor signaling. Given the evidence implicating Cas and CasL as important signaling intermediates downstream of these receptors, it is important to address the role of Sin and Sin isoforms in this receptor system.

Role of Sin in Src kinase signaling

Given that Sin was isolated as a protein that binds to Src-kinase SH3 domains, we previously examined the functional properties of Sin in relation to Src kinases (61). The enzymatic activity of Src kinases is regulated intramolecularly by the conserved Src SH3 and SH2 domains (79–82). The SH3 domain inhibits Src kinase activity by binding to a 14 amino acid linker region located between the SH2 and kinase domains of the molecule (*Fig.* 3) (83, 84). The SH2 domain contributes to this inhibition by binding to a conserved, phosphotyrosine-containing motif at the C-terminus of Src (79, 83, 84). Mutations that disrupt these interactions lead to constitutively active Src enzymes and cellular transformation.

In our experiments, we used Sin as a high-affinity ligand for the Src-SH3 domain to outcompete these intramolecular interactions and activate Src enzymatic activity and signaling (*Fig.* 3, *step* 1) (55, 61). We found that whereas full-length Sin only moderately activates Src, a C-terminal deletion mutant of Sin, SinΔC (*Fig.* 2A), is a better inducer of Src kinase activity, mediates signaling and becomes highly phosphorylated on conserved tyrosine residues in an Src kinase-dependent manner (*Fig.* 3, *step* 1). As mentioned earlier, Sin and the other Cas proteins have tyrosine-based motifs that exhibit specificity for the Crk-SH2 domain (*Table* 2). Through mutagenesis analysis

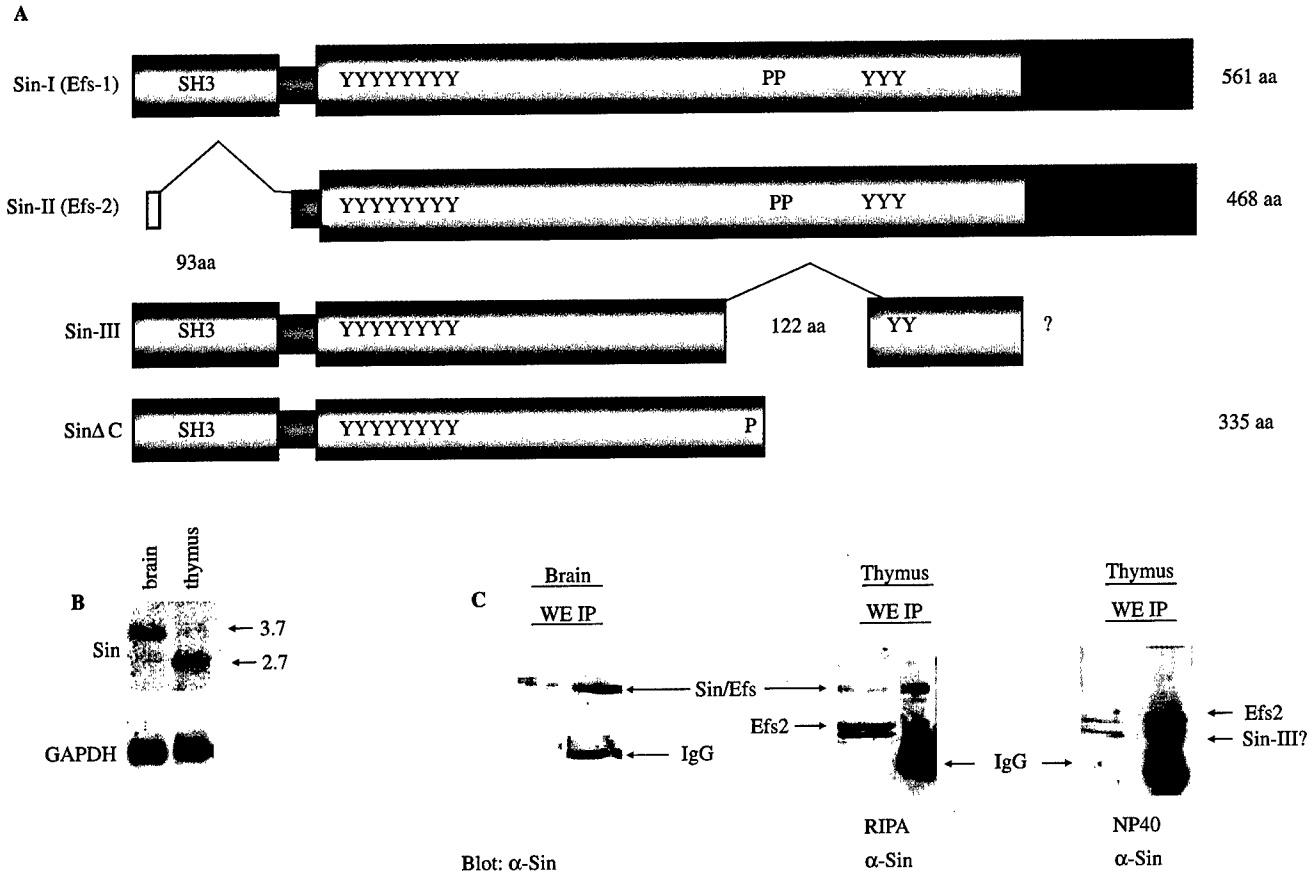

Fig. 2. Schematic representation of Sin isoforms and expression of Sin in the thymus and brain. (A) The structures of Sin isoforms: full-length Sin, Efs2, which contains a deletion of 93 amino acids encompassing the SH3 domain, and Sin III, a partial cDNA clone isolated from a mouse thymus cDNA library containing an internal deletion of 122 amino acids. SinΔC represents an activated deletion mutant that was used in previous experiments to study the function of Sin (55, 61). **(B)** Northern blot of total RNA probed with a Sin-specific probe. Total RNA (10 μg) from mouse brain and thymus was run on an agarose gel, transferred to nitrocellulose and probed with a Sin cDNA fragment corresponding to amino acids 244–335 (55) and exposed on film for 2 days. The membrane was then stripped and reprobed with GAPDH cDNA to normalize for RNA content. Two different RNA species that exhibit differential expression patterns between brain and thymus are shown. **(C)** Brain and thymus cell extracts were lyzed in 0.1 sodium dodecyl sulphate (SDS) Radio immuno precipitation assay buffer (RIPA) or 1 NP-40-containing buffers as shown, and cell lysates were normalized for protein content. Extracts were then immunoprecipitated (IP) with Sin-specific antibody, immune complexes separated on SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and Western blotted with Sin-specific antibody. Total cell extracts (WE), also normalized for protein content, were included on the blots as controls (58). Protein bands were visualized by enhanced chemiluminescence (ECL; Amersham).

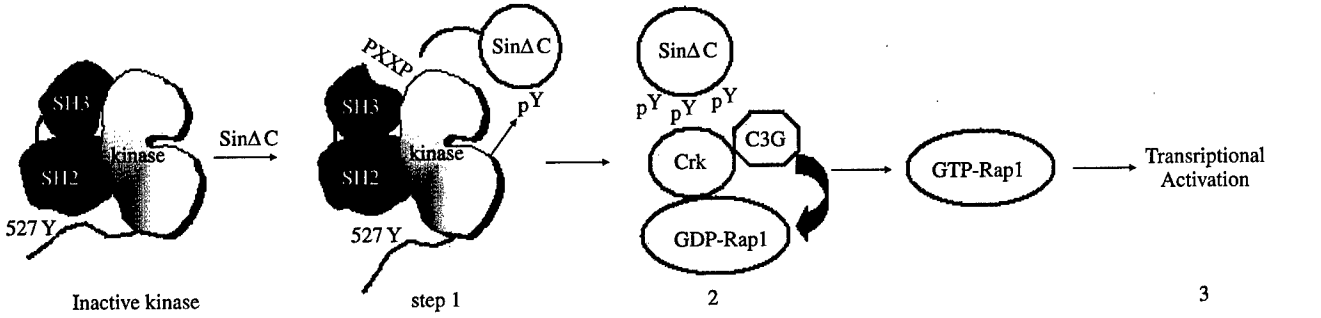

Fig. 3. Model for truncated Sin-induced Src activation and signaling. Coexpression of SinΔC and Src kinases in 293HEK cells leads to interaction of the Sin proline-rich motifs with the Src-SH3 domain. This leads to increased Src kinase activity and SinΔC phosphorylation (step 1). Phosphorylated SinΔC then recruits the Crk/C3G complex through specific phosphorylated tyrosine motifs, which in turn activates Rap1 (step 2). In this cell system, activation of Rap1 leads to ERK phosphorylation and transcriptional activation (step 3) (61).

we identified three phosphotyrosine residues (Y148, Y188, and Y253) that bind to the adapter Crk and are important for the ability of Sin to mediate Src signaling (*Fig. 3, step 2*). In cells coexpressing Src and SinΔC, SinΔC is found in a complex with Crk and C3G, a protein known to act as a GEF for Rap1 (*Fig. 3, step 2*). Consistently, we found that SinΔC-mediated Src signaling is dependent on the small GTP-binding protein Rap1 (*Fig. 3, step 3*). In addition, using dominant negative inhibitors of Crk and Rap1, we were able to show that these proteins indeed are required for SinΔC-mediated Src signaling. In the same experiments, we found no involvement of the related Ras protein downstream of SinΔC-activated Src (61). These experiments for the first time identified Rap1 as a signaling mediator acting downstream of Src kinases and provided clues to the function of Sin.

Role of Sin in thymocyte development

To gain insight into the physiologic properties of Sin in relation to Src kinases, we examined the role of Sin in T lymphocyte function. We used T lymphocytes as our model system, because the thymus is one of the tissues in which endogenous Sin is most highly expressed (*Fig. 2B,C*). In addition, Sin is an Src kinase substrate, and two Src kinases, Fyn and Lck, are known to play important roles in T lymphocyte physiology (85–87). Because in previous experiments we found that the truncated protein SinΔC (61) more efficiently binds to and becomes phosphorylated by Src kinases, we used this protein for our studies. Using transgenic mice, we found that specific expression of SinΔC in thymocytes resulted in reduced cell numbers (40% of wild-type) evidenced by an overall reduction in the size of the thymus (58). This phenotype correlated with decreased thymocyte survival and increased SinΔC-mediated thymocyte apoptosis assayed by AnnexinV and 7AAD staining. We also found that thymocyte maturation was compromised in SinΔC-expressing animals. Thus, whereas the percentages of the precursor CD4 and CD8 double-negative ($CD4^-CD8^-$,DN) and double-positive ($CD4^+CD8^+$,DP) populations were normal, we found reduced percentages of mature $CD4^+$ and $CD8^+$ single-positive (SP) T cells (60% and 90% inhibition, respectively) (58). These data are summarized schematically in *Fig. 4*.

We found that SinΔC is consistutively phosphorylated by the Src kinase Fyn but not by the related kinase Lck (58). Crossing the SinΔC mice to $fyn^{-/-}$ animals revealed that the expression of Fyn is required for SinΔC-mediated thymocyte apoptosis but not for SinΔC-mediated inhibition of thymocyte maturation (*Fig. 4*). Consistent with the SinΔC-induced block in thymocyte maturation even in the absence of Fyn, we found reduced but significant phosphorylation of SinΔC in the $fyn^{-/-}$ background (58). This observation suggests that SinΔC is phosphorylated by tyrosine kinases other than Fyn, which regulate the ability of Sin to inhibit thymocyte maturation. As Sin contains a tyrosine motif that can potentially bind to the Itk-SH2 domain (*Table 2*), it is possible that Tec kinases may also be involved in Sin phosphorylation. This possibility is currently under investigation.

The inhibitory effect of SinΔC on thymocyte maturation correlated with defective activation of the extracellular signal regulated kinase (ERK) MAP kinase, which has been shown to play an important role in thymocyte selection (58). In the same experiments, activation of the JNK kinase was intact, suggesting that the effect of SinΔC on ERK was specific and that SinΔC may inhibit T cell maturation by interfering with normal selection of thymocytes. Experiments addressing the effect of SinΔC on thymocyte positive and negative selection using TCR transgenic animals are currently under way. Expression of SinΔC had no effect on upstream phosphorylation events proximal to the TCR, suggesting that SinΔC expression does not interfere with Src kinase (Fyn/Lck) phosphorylation of substrates, which is required for normal signaling, and that the effect of SinΔC is downstream of these phosphorylation events (58). From these experiments, we conclude that the truncated Sin mutant inhibits thymocyte differentiation through Fyn-dependent and -independent mechanisms and that endogenous Sin may be an important regulator of T lymphocyte development.

Role of Sin in T cell activation

Consistent with the reduction in the SP T cell populations in the thymi of transgenic animals, we also found reduced numbers of mature $CD4^+$ and $CD8^+$ T cells in the spleen of transgenic animals. Thus, the number of mature $CD3^+$ SP transgenic T cells is approximately 50% of normal littermate controls (*Fig. 4*). The B cell population, on the other hand, is unaffected, given the CD2 promoter-driven, T cell-specific expression of SinΔC. T cells purified from the spleens of transgenic animals fail to proliferate and do not produce IL-2 in response to TCR stimulation, suggesting that SinΔC expression interferes with TCR signaling that leads to IL-2 expression (*Fig. 5A*, and unpublished observations).

We further analyzed the mechanism of SinΔC-mediated inhibition of T cell proliferation and whether SinΔC reflected the function of full-length Sin in Jurkat T cells. To this end we used Jurkat T cell lines stably overexpressing Sin or cells

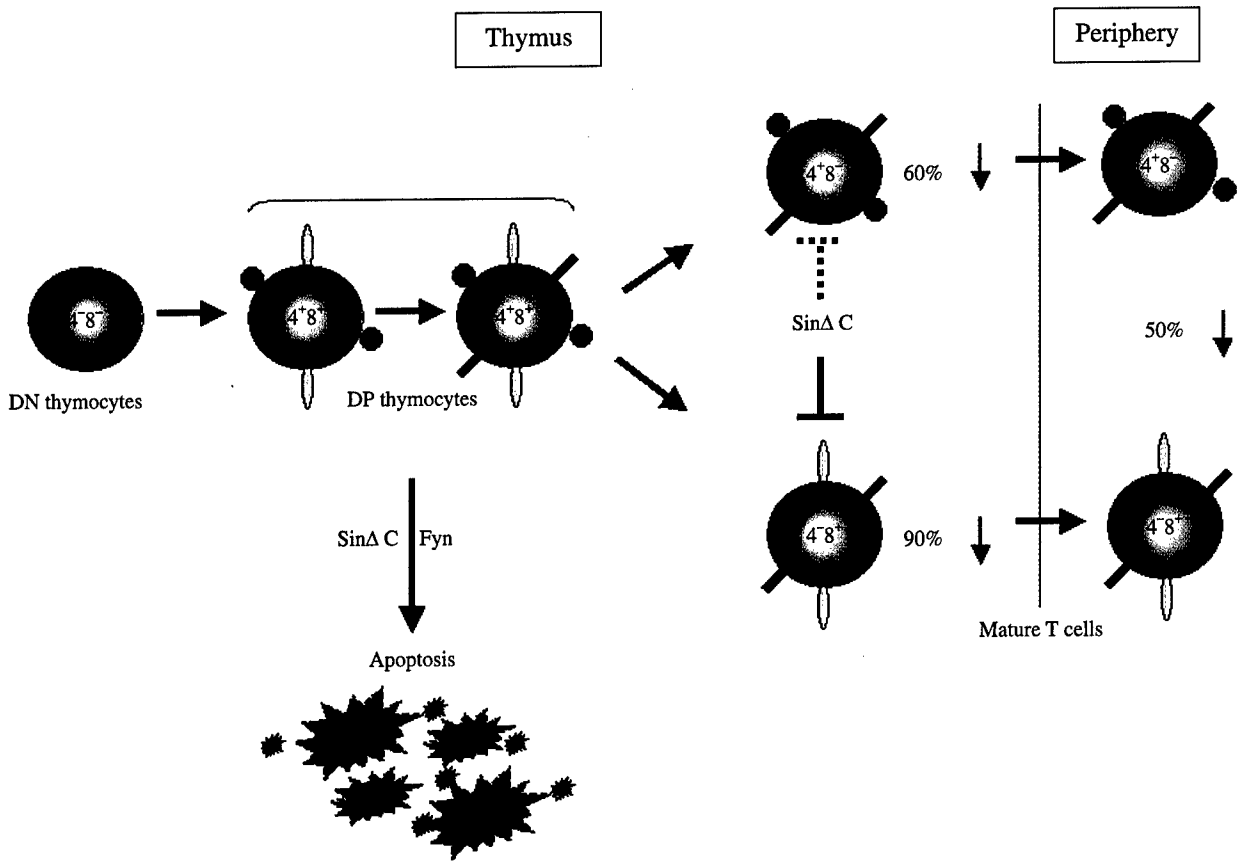

Fig. 4. Effect of SinΔC expression on thymocyte development. Expression of the truncated Sin mutant reduces thymic cellularity by inducing thymocyte apoptosis in a Fyn-dependent manner. On the other hand, Fyn-independent SinΔC-mediated inhibition of T cell maturation leads to reduced percentages of $CD4^+$ and particularly $CD8^+$ SP cells in the thymus as well as in the spleen.

transiently transfected with Sin or SinΔC together with luciferase reporter constructs. These constructs contain DNA binding sites for nuclear factor of activated T cells (NFAT) and AP-1 transcription factors from the IL-2 promoter as well as the complete IL-2 transcriptional unit. We found that both the full-length protein and SinΔC inhibit TCR-induced NFAT and AP-1 activation, consistent with the defect in proliferation and IL-2 production we observe in transgenic SinΔC-expressing T cells (Xing et al., in preparation) (*Fig. 5A*). Sin expression very effectively inhibits NFAT activation (~80%), whereas the Sin-mediated AP-1 inhibition is partial (~50%). The effects of Sin and SinΔC on transcriptional inhibition are similar, suggesting that both proteins behave as negative regulators of TCR-stimulated T cell activation and that SinΔC reflects the function of endogenous Sin.

To further analyze the inhibitory effects of Sin on transcriptional activation, we examined the mechanisms of Sin-mediated inhibition on NFAT and AP-1 activation. We first examined the effect of Sin on AP-1-mediated transcription. The AP-1 transcription factor consists of heterodimers of the Fos and Jun proteins, whose expression and activation is regulated by the upstream MAP kinases ERK and JNK. Consistent with the inhibitory effect of Sin on AP-1-dependent transcriptional activation, we found that ERK phosphorylation in response to TCR crosslinking was compromised in SinΔC-expressing transgenic and Sin overexpressing T cells. In contrast, JNK phosphorylation was normal, suggesting that the partial inhibitory effect of Sin on AP-1-mediated gene expression was the result of defective ERK, but not JNK, activation (Xing et al., in preparation).

The defect in ERK activation appears to be the result of a downstream inhibitory event, as phosphorylation of substrates proximal to the TCR appeared normal in SinΔC transgenic and Sin-expressing Jurkat T cells, consistent with our results in thymocytes (58). The nature of this inhibitory event is not clear at present. However, it has been shown that, in T cells, regulation of phosphorylation of MAP kinases is modulated by the GTPases Ras and Rap1. Whereas Ras plays a positive role in MAP kinase activation, Rap1 can play an inhibitory role in T cell activation and proliferation. In fact, suboptimally

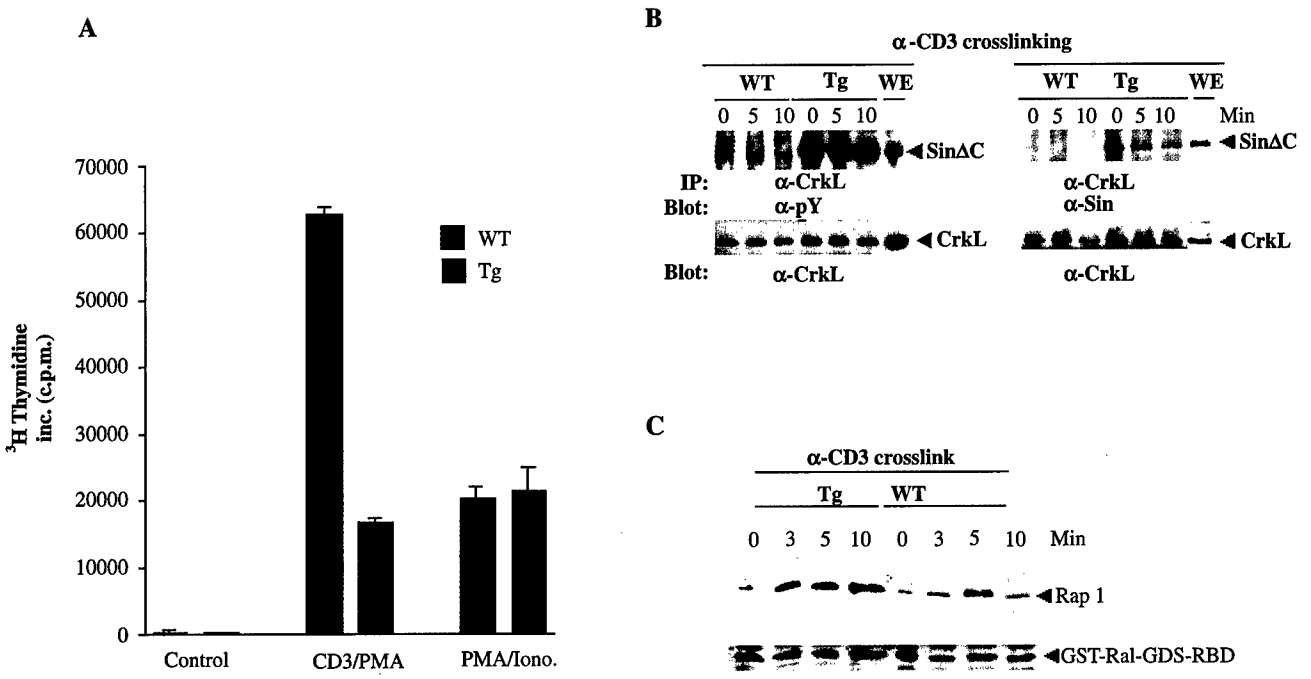

Fig. 5. SinΔC-mediated inhibition of proliferation correlates with Rap1 activation. (A) Purified T cells (1×10^5) isolated from the spleens of SinΔC transgenic animals were left untreated (control) or stimulated with CD3/PMA (0.5 μg/mL) or PMA/ionomycin (50 ng/mL/1 μM). After 2 days in culture, cells were labeled with ^{3}H thymidine (1 μCi) for 18 h, cells were then harvested and incorporation of radiolabeled thymidine was determined using a scintillation counter. **(B)** Splenocytes (1×10^7 per sample) from wild-type or transgenic animals were stimulated with 2 μg CD3-specific antibody for the indicated times. Cell extracts were then immunoprecipitated with antibody against the lymphocyte-specific isoform of Crk, CrkL, immune complexes were separated on SDS-PAGE, transferred to nitrocellulose membranes and Western blotted with antiphosphotyrosine- (left panel) or Sin-specific (right panel) antibodies. The membranes were then stripped and reprobed with CrkL-specific antibody. Protein bands were visualized by ECL. WE represents whole cell extracts. **(C)** Splenocytes were stimulated as in **(B)** with an antibody against CD3, lysed and GTP-bound Rap1 was precipitated with a GST-fusion protein containing the Rap1-binding domain of Ral-GDS-RBD as described previously (61). GTP-Rap1 was visualized using a Rap1-specific antibody. The nitrocellulose membranes were stripped and reprobed with anti-GST antibody to visualize GST-Ral-GDS-RBD to control for equal loading.

stimulated T cells (without costimulation through CD28 coreceptor) exhibit constitutive Fyn activity and increased levels of GTP-Rap1 (42). This correlates with inhibition of IL-2 production and T cell proliferation (42). Based on this and other studies (88–90) it has been proposed that in certain cell types, such as T lymphocytes, Rap1 activation plays an inhibitory role in cellular function by interfering with normal Ras signaling and ERK activation. However, more recent evidence suggests that Rap1 plays a positive role in thymocyte development and T cell activation and that TCR-activated Rap1 does not interfere with positive selection and ERK phosphorylation (91).

We mentioned earlier that in a cell culture system Src-phosphorylated SinΔC binds to the adapter Crk and the nucleotide exchange factor C3G, both upstream activators of Rap1 (61). We have also shown that SinΔC mediates Src signaling through the activation of the small GTP-binding protein Rap1, whose activation leads to increased phosphorylation of ERK and activation of transcription (61). In SinΔC-expressing thymocytes, we have observed constitutive association of phosphorylated SinΔC with the lymphocyte-specific isoform of Crk, CrkL, and increased Rap1 activation compared to normal cells (*Figs* 5B,C). It is thus possible that Sin expression leads to activation of Rap1 through CrkL recruitment, which in turn interferes with ERK phosphorylation. In addition, we have evidence that Sin binds to the thymus-specific isoform of the GEF Chat, Chat-H (as mentioned earlier), which was previously shown to bind to the Ras family of proteins Rap1 and R-Ras. Moreover, AND-34, a molecule related to Chat-H, binds to and promotes guanine nucleotide exchange on Ral, Rap1 and R-Ras. Thus, Sin can potentially modulate the GTP levels of Ras proteins and subsequent phosphorylation of MAP kinases in T cells through two different mechanisms; that is, through the recruitment of the CrkL/C3G complex and through association with Chat-H. Modulation of the relative GTP levels of the Ras and Rap1 proteins through these mechanisms may thus lead to inhibition of ERK phosphorylation. Given the conflicting evidence about the role of

Rap1 in T lymphocytes, further experiments are required to address the role of Rap1 in inhibition of ERK phosphorylation in our system.

We subsequently examined the mechanisms of Sin-mediated NFAT transcriptional activation. Given that NFAT-activation is regulated through PLC-γ-induced intracellular calcium release, we examined the effect of Sin on intracellular calcium mobilization in response to TCR stimulation in Jurkat T cell lines stably transfected with Sin. Consistent with the defective NFAT activation we observed in the luciferase reporter assays, we found that Sin expression inhibited intracellular calcium release as compared to T cells stably transfected with a control vector. These results suggested that Sin expression blocks NFAT activation by interfering with intracellular calcium mobilization. As increases in the intracellular calcium concentrations are regulated by TCR-induced PLC-γ activation, we also examined whether PLC-γ phosphorylation was compromised in the presence of Sin. Consistent with our observation that Sin-overexpressing cells exhibit defective calcium mobilization, we found that expression of Sin in Jurkat T cells correlated with inhibition of PLC-γ phosphorylation in response to TCR crosslinking (Xing et al. in preparation). These results suggest that Sin blocks NFAT activation and, as a result, IL-2 production by inhibiting PLC-γ activation and intracellular calcium release.

The mechanism of Sin-mediated inhibition of PLC-γ is not yet clear. As shown in *Table 2*, Sin contains conserved motifs that can act as potential binding sites for the SH2 and SH3 domains of PLC-γ. Experiments are in progress to determine the *in vivo* association of endogenous and overexpressed Sin with PLC-γ under resting and TCR-stimulated conditions. Our hypothesis is that endogenous Sin regulates TCR signaling by setting a threshold of activation for, and regulating the activity of, signaling intermediates and that overexpression of Sin reveals this inhibitory function. A similar mechanism has been described previously for the adapter molecule Cbl in the regulation of immune receptor signaling. It has been shown that Cbl binds to and inhibits the phosphorylation and activation of the tyrosine kinase Syk in response to FcεRI receptor stimulation in mast cells, effectively blocking degranulation (43). Sin could be acting in a similar fashion by binding to PLC-γ and inhibiting its association with LAT and/or its phosphorylation by ZAP70 and Itk, which are both required for the induction of phospholipase enzymatic activity (14). As all Cas proteins contain potential binding sites for Itk (*Table 2*), it would be of interest to determine whether Itk binds to Sin and whether that affects PLC-γ phosphorylation. These possibilities are currently under investigation.

Concluding remarks

We have reviewed evidence pertaining to the function of the three structurally related proteins of the Cas family. Although very limited information exists about the function of p130Cas in T lymphocytes, evidence generated in our laboratory as well as by other groups suggests that Sin and CasL (54) are both regulators of T cell signaling and activation. However, while existing evidence suggests that CasL is a positive regulator of T cell signaling, we found that Sin has the opposite effect and acts as a negative regulator of T cell proliferation. Given the structural similarity of Sin and CasL and their potential to bind to some common intermediates (*Table 1*), the existing data thus far argue against functional similarity and overlap. Additional evidence supporting functional divergence for Sin and CasL is the observation that, whereas CasL is transiently phosphorylated in peripheral blood T cells and Jurkat cells overexpressing full-length CasL, we have not been able to detect increased Sin phosphorylation in Jurkat cells stably overexpressing Sin. In contrast, in the same cell system we find that overexpressed Sin is constitutively phosphorylated and becomes rapidly and transiently dephosphorylated in response to TCR crosslinking (unpublished observations). A similar mechanism of regulating the ability of scaffold molecules to assemble protein complexes has been reported recently in the case of the PAG/Cbp protein. TCR-induced dephosphorylation of PAG/Cbp results in the release and relocalization of Csk to the cytosol and activation of Src kinases. Experiments are in progress to determine whether this reflects endogenous regulation of Sin phosphorylation.

The differential in the phosphorylation patterns of Sin vs. CasL could be due to structural differences, including the presence of proline-rich motifs on Sin but not on CasL. These motifs can influence the binding of kinases, Src kinases in particular, and as a consequence affect the mode of phosphorylation of the two proteins. Consistent with the idea of differential binding of Src kinases are the observations that although Fyn and Lck both bind to and phosphorylate CasL in response to the TCR (77), we only find association of Sin with Fyn and not with Lck (58). The fact that the Src kinases interact with CasL in the absence of proline-rich motifs also supports the existence of differential mechanisms for recruiting Src kinases by Sin vs. Cas.

In addition to the different biochemical properties of Sin and CasL, the expression patterns of Sin also provide evidence in support of functional divergence between Sin and Cas. Our data show that at least two other Sin proteins (Sin II and III) are expressed in the thymus in the form of alternatively spliced isoforms of the full-length protein. To our knowledge, no

splice variants of CasL and p130Cas have yet been described. The expression patterns of Sin in the thymus vs. the brain are also different, since we have not yet been able to detect the spliced isoforms of Sin in the adult mouse brain. The existence of the Sin III isoform is particularly intriguing, given that the introduced deletion removes the two proline-rich motifs that bind to Src kinase SH3 domains as well as one of the Sin tyrosine motifs (*Fig. 2A*). Collectively, these observations argue for unique roles for the CasL/Sin proteins in T lymphocyte function and T cell physiology. We anticipate that the generation of Sin null animals will provide important insights into the role of Sin in T cell physiology.

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Defective Thymocyte Maturation by Transgenic Expression of a Truncated Form of the T Lymphocyte Adapter Molecule and Fyn Substrate, Sin[1]

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Adapter molecules that promote protein-protein interactions play a central role in T lymphocyte differentiation and activation. In this study, we examined the role of the T lymphocyte-expressed adapter protein and Src kinase substrate, Sin, on thymocyte function using transgenic mice expressing an activated, truncated allele of Sin (SinΔC). We found that SinΔC expression led to reduced numbers of $CD4^+$ and $CD8^+$ single-positive cells and reduced thymic cellularity due to increased thymocyte apoptosis. Because the adapter properties of Sin are mediated by tyrosine-based motifs and given that Sin is a substrate for Src tyrosine kinases, we examined the involvement of these kinases in the inhibitory effects of SinΔC. We found that in transgenic thymocytes, SinΔC was constitutively phosphorylated by the Src kinase Fyn, but not by the related kinase Lck. Using SinΔC and $fyn^{-/-}$ animals, we also found that the expression of Fyn was required for the inhibitory effect of SinΔC on thymocyte apoptosis but not for SinΔC-mediated inhibition of T cell maturation. The inhibitory effect of SinΔC on thymocyte maturation correlated with defective activation of the mitogen-activated protein kinase extracellular signal-regulated kinase. Our results suggest that the Sin mutant inhibits thymocyte differentiation through Fyn-dependent and -independent mechanisms and that endogenous Sin may be an important regulator of thymocyte development. *The Journal of Immunology,* **2002, 169: 6900–6909.**

In recent years, adapter proteins have been shown to play important roles in the signaling mechanisms of a variety of cell types including T and B lymphocytes. Adapter proteins by definition lack enzymatic or transcriptional activity and control cellular behavior by mediating constitutive or inducible protein-protein or protein-lipid interactions through modular interaction domains (1–6). T lymphocytes express a variety of adapter molecules that act as positive or negative regulators of TCR-dependent signaling. Positive regulators include linker for activation of T cells, Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP76),[3] and Grb2-related adapter downstream of Shc whereas negative regulators include Casitas B cell lymphoma (Cbl), SLP76-associated protein, protein associated with glycosphingolipid-enriched microdomains/CSK-binding protein (2–7). The important roles of these adapters in T lymphocyte function have been substantiated with gene targeting experiments showing that SLP76, Grb2-related adapter downstream of Shc, and linker for activation of T cells are absolutely required for normal thymocyte development (8–10). In contrast, mice deficient for negative regulators of TCR signaling such as c-Cbl, Cbl-b, and Src-like adapter protein exhibit opposite phenotypes. These phenotypes include increased positive selection, increased expression of the surface molecules CD3, CD4, and CD5, markedly up-regulated activity of ζ-associated protein of 70 kDa, and extracellular signal-regulated kinase (ERK) (11–13), as well as spontaneous autoimmunity due to reduced T cell activation thresholds (14, 15).

Our previous studies have concentrated on addressing the role of the novel adapter molecule Sin in the signaling pathways of Src family nonreceptor tyrosine kinases (16). Sin/Efs1 was cloned as a high affinity ligand for the Src- and Fyn-Src homology (SH)3 domains (16, 17) and belongs to a family of proteins, the other members being $p130^{Cas}$ and human enhancer of yeast filamentation 1/Crk-associated substrate in lymphocytes (18), (19–22). The adapter properties of Sin and the other family members are due to conserved sequence motifs that mediate protein-protein interactions. These conserved motifs consist of: 1) proline-rich sequences that bind to SH3 domains such as those found on Src kinases (19, 23); 2) conserved tyrosine residues which, when phosphorylated by Src and other kinases, mediate interactions with SH2-domain-containing substrates (24); and 3) an SH3 domain that is highly conserved among the three family members (19).

Given that Sin was isolated as a protein that binds to Src-kinase SH3 domains, we previously examined its functional properties in relation to Src kinases. The enzymatic activity of Src kinases is regulated intramolecularly through interactions of the conserved Src SH3 and SH2 domains with specific sequences within the Src

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Received for publication July 18, 2002. Accepted for publication October 15, 2002.

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[1] This work was supported in part by American Cancer Society Grant RPG99-09-01 MGO, Department of Defense Grants DAMD17-99-1-9151 and DAMD17-99-1-915, National Institute of Allergy and Infectious Diseases Grant RO1 AI49387-01, American Cancer Society Institutional Research Grant No. 177F, and the Herbert Irving Comprehensive Cancer Center. L.T.D. was supported by National Institutes of Health Grant 5 T32 AI07525. A.G.R. was supported by National Institutes of Health Grant 5 T32 GM03767-26. K.A. was supported in part by Department of Defense Grants DAMD17-99-1-9151 and DAMD17-99-1-915, National Institute of Allergy and Infectious Diseases Grant RO1 AI49387-01, and American Cancer Society Grant RPG99-09-01 MGO.

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[3] Abbreviations used in this paper: SLP76, Src homology 2 domain-containing leukocyte protein of 76 kDa; Cbl, Casitas B cell lymphoma; ERK, extracellular signal-regulated kinase; SH, Src homology; SP, single positive; JNK, jun N-terminal kinase; 7AAD, 7-amino actinomycin D; MAPK, mitogen-activated protein kinase; DN, double negative; DP, double positive.

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kinase molecules (25–28). Mutations that disrupt these interactions lead to constitutively active Src proteins and cellular transformation. In our experiments, instead of Src-constitutively active mutants, we used Sin as a high affinity ligand for the Src-SH3 domain, to outcompete the inhibitory intramolecular interactions of Src and activate the enzyme (16). We found that whereas full-length Sin only moderately induced Src enzymatic activity and signaling, a C-terminal deletion mutant of Sin, SinΔC, was a potent activator of Src kinase activity and signaling (29).

The physiologic function of Sin is currently not known. In this report, we examined the role of Sin in T lymphocyte function because the thymus is one of the tissues in which endogenous Sin is most highly expressed. In addition, Sin is a Src kinase substrate and two Src kinases, Fyn and Lck, are known to play important roles in T lymphocyte physiology (30–32). Given that Sin-mediated protein-protein interactions are facilitated by Src kinase-phosphorylated tyrosine motifs (29, 33), we thought it was important to address the function of Sin in relation to these kinases. In previous experiments we found that the truncated form of Sin, SinΔC, was a better activator of and was more efficiently phosphorylated by Src kinases than the full-length Sin (29). Therefore, we used this protein for our studies. We found that specific expression of SinΔC in thymocytes of transgenic animals inhibited thymocyte development and survival, shown by reduced percentages of mature $CD4^+$ and $CD8^+$ single-positive (SP) T cells and increased thymocyte apoptosis. We also found that Sin was a substrate for Fyn but not Lck, and that Fyn was required for Sin-mediated thymocyte apoptosis but not for the block in thymocyte maturation. Moreover, SinΔC expression correlated with reduced ERK activation, which is required for proper selection of thymocytes. These experiments are the first to address the role of Sin in T lymphocyte function in vivo and suggest that truncated Sin is a negative regulator of T lymphocytes through Fyn-dependent and -independent mechanisms and that endogenous Sin may be an important regulator of T lymphocyte function.

Materials and Methods

Generation of transgenic mice

A cDNA fragment encoding aa 1–335 of full-length Sin was cloned into the *Eco*RI/*Sma*I site of the CD2 expression cassette containing the human CD2 minigene previously described to express the transgene both in immature thymocytes and in mature T cells (34). An 11.2-kb *Kpn*I/*Not*I fragment containing the transgene was microinjected into (C57BL/6 × CBA/J)F_1 fertilized eggs derived from hyperovulated donor females. Transgenic founders were identified by PCR and Southern blot analysis of tail DNA and transgenic lines were established by backcrossing to C57BL/6 mice.

Mice

C57BL/6 and *fyn*$^{-/-}$ animals were purchased from The Jackson Laboratory (Bar Harbor, ME).

Abs and reagents

Mouse mAbs: anti-Sin-specific Ab was obtained from BD Transduction Laboratories (Lexington, KY); anti-Fyn, anti-Lck, and anti-phospho-ERK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphotyrosine-specific Ab was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-jun N-terminal kinase (JNK), and phospho-JNK were obtained from New England Biolabs (Beverly, MA) and anti-ERK goat polyclonal was obtained from Santa Cruz Biotechnology. Dexamethasone and enolase were purchased from Sigma-Aldrich (St. Louis, MO).

Flow cytometry

Freshly isolated thymocytes (1×10^6) from 6- to 8-wk-old mice were incubated with the appropriate Abs in staining medium (3% FCS, 0.1% sodium azide in PBS) for 15 min on ice. Cells were spun down and washed three times with staining medium and analyzed by flow cytometry using a FACSCalibur and CellQuest software. Anti-CD4-allophycocyanin, CD8-PerCP, CD3-FITC, CD69-FITC, CD5-FITC, TCR-β-FITC-conjugated Abs were purchased from BD PharMingen (San Diego, CA). Purified mouse monoclonal anti-CD3 Ab used for cross-linking was obtained from BD PharMingen.

Apoptosis assays

For analyzing spontaneous thymocyte apoptosis in vitro, 5×10^5 thymocytes from SinΔC transgenic and negative littermate controls were cultured in tissue culture medium for different time points. Dexamethasone (10 μM) was added in parallel cultures as a control for apoptosis. Thymocyte apoptosis was assayed by Annexin V^{PE} and 7-amino actinomycin D (7AAD) staining, using an apoptosis detection kit (BD PharMingen) according to the manufacturer's protocol and a FACSCalibur with CellQuest software. Cell debris was gated out on the basis of forward and side scatter analysis. Percent apoptosis is expressed as the amount of cells staining positive for annexin V and negative for 7AAD (early stage) and survival is represented as percent of cells that were annexin V- and 7AAD-negative.

TCR cross-linking

Thymocytes (1×10^7) were incubated with 2 μg of anti-CD3 Ab on ice for 15 min, washed with cold PBS, and supercross-linked with 5 μg of goat-anti-mouse IgG for 20 min on ice. Cells were then incubated at 37°C for different points, spun down, and immediately lysed. Cell lysates were used for immunoblot analysis.

Mitogen-activated protein kinase (MAPK) assays

Thymocytes ($0.5–1 \times 10^7$) were cross-linked with anti-CD3 for different time points. Total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-phospho-ERK or anti-phospho-JNK to reveal the phosphorylated forms of these kinases. Blots were stripped and reprobed with anti-ERK and anti-JNK Abs to determine the amounts of total ERK and JNK.

Immunoprecipitations

Immunoprecipitations were performed as previously described (16). Briefly, cells were lysed in 1 ml of ice-cold Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and incubated on ice for 30 min. The cell debris and nuclei were removed by centrifugation for 10 min at 4°C. The cell lysates were then incubated with the specified Abs at concentrations suggested by the manufacturers for 2 h at 4°C. The immune complexes were collected after the addition of 20 μl of protein G- plus protein A-agarose (Oncogene Research Products, San Diego, CA) and incubation at 4°C for 30 min. The pellets of agarose beads were washed three times with 1 ml of lysis buffer and then subjected to SDS-PAGE and immunoblotting.

In vitro kinase assays

Protein complexes obtained by immunoprecipitation were washed three times in kinase buffer and reactions were conducted in 20 μl of kinase buffer containing 20 mM HEPES, pH 7.4, 5 mM MnCl, 10 μM ATP, and 1 μl of [γ-^{32}P]ATP (5000 Ci/mmol) at room temperature for 5 min. When required, 5 μg of the exogenous substrate enolase was added to the samples as shown and as previously described (35). The pellets were resuspended in 1× Laemmli buffer, boiled for 5 min, and phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

Western blot analysis

Total cell extracts or immunoprecipitates normalized for protein content were boiled in Laemmli sample buffer, electrophoretically separated on 10% SDS-PAGE, and transferred to nitrocellulose membranes. Filters were blotted with the appropriate monoclonal antisera according to manufacturer's protocol in TBST/milk at 4°C overnight (16 h). Rabbit polyclonal Abs were used at a 1/500 dilution. mAbs were used at 1 μg/ml TBST/milk each. The filters were washed in TBST and consequently incubated with anti-mouse or anti-rabbit IgG-conjugated HRP at a 1/4000 dilution in TBST at room temperature for 1 h. Filters were then washed and developed with ECL (Amersham, Piscataway, NJ), as described by the manufacturer.

Results

Generation of SinΔC-expressing transgenic animals

On Northern blots endogenous Sin is most highly expressed in the adult thymus and brain, while Sin can be detected in multiple tissues by RT-PCR (36). At least two Sin isoforms can be detected

in thymocyte and T cell extracts on Western blots of total lysates and Sin immunoprecipitates; full-length Sin and the previously described Efs2 (36), a Sin alternative splice form in which the SH3 domain is deleted (Fig. 1, *A*, *B*, and *D*) (36).

To explore the function of Sin in T lymphocytes, we generated transgenic mice expressing the truncated form of Sin, SinΔC (Fig. 1*A*). The truncation removes a C-terminal portion of Sin and exposes one of the proline-rich motifs of Sin that binds to Src kinase SH3 domains with high affinity. We have previously shown that the C terminus of Sin inhibits the ability of Sin to mediate Src signaling. Deletion of this region results in a protein that is a better activator and transducer of Src kinase signaling than the full-length Sin protein (29). In the experiments described in this study we used this activated mutant because efficient Src kinase-mediated phosphorylation of tyrosine residues within Sin is required for Sin adapter function (29). SinΔC expression in the transgenic animals is regulated by the human CD2 promoter, which allows transgene expression in both thymocytes and mature T cells (34).

Transgenic progeny from three founder mice were identified by PCR and Southern blot analysis and expression of the transgene was confirmed in thymocyte extracts using a Sin-specific Ab (Fig. 1*C*). Two transgenic lines CR1 and MA2 with 22 and 13 integrated copies of the transgene, respectively, were chosen for further studies. Expression of the transgene in CR1 mice was compared with the endogenous full-length Sin and Efs2 proteins on Western blots of total thymocyte lysates probed with Sin-specific Ab as shown (Fig. 1*D*). Expression of Efs2 is more pronounced than full-length Sin and in transgenic lysates Efs2 migrates slightly above SinΔC. The physiologic significance for the presence of two different isoforms is currently not clear and is under investigation.

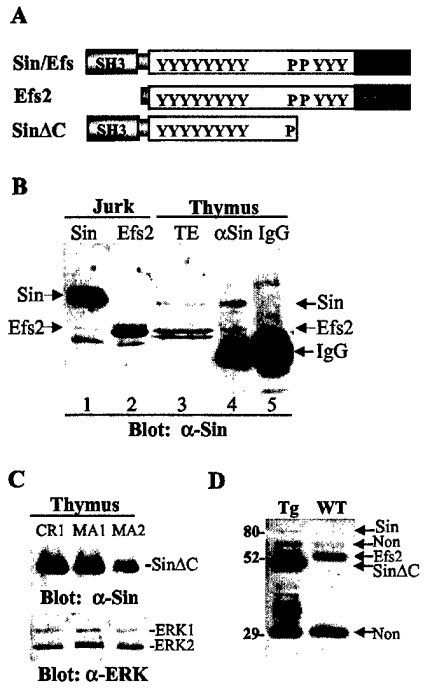

FIGURE 1. Two different Sin isoforms are expressed in the thymus. *A*, Schematic representation of Sin isoforms and SinΔC. Full-length Sin consists of a SH3 domain, a central region containing eleven tyrosine residues, and two proline-rich motifs (RPLPALP and RPLPPPP) that have high binding affinity and specificity for Src kinase SH3 domains. Efs2 is a naturally occurring isoform that is missing 93 aa consisting of the entire SH3 domain and additional residues before the central substrate-binding region of the protein. SinΔC is missing an ~200 aa fragment, which contains the second proline-rich motif (RPLPPPP), three tyrosine residues, and a conserved region of ~140 aa at the extreme C terminus. Y represents tyrosine-containing sequences (Y motifs), P represents proline-rich motifs. *B*, Western blots of thymocyte and Jurkat T cell extracts probed with a Sin-specific mAb to reveal expression of the Sin isoforms. *Lanes 1* and *2* represent total cell lysates from Jurkat cells overexpressing full-length Sin and Efs2 as controls. TE represents total thymocyte extract (*lane 3*) probed with anti-Sin-specific Ab. In *lanes 4* and *5*, cell extracts from 10^7 thymocytes were immunoprecipitated with anti-Sin or isotype-matched control IgG, proteins were separated on SDS-PAGE and Western blotted with Sin-specific Ab. *C*, Thymocyte cell extracts from three different founders expressing SinΔC were separated on SDS-PAGE and Western blotted with anti-Sin Ab. The blot was stripped and reprobed with ERK-specific Ab as a loading control. *D*, Endogenous Sin expression was compared with expression of SinΔC from CR1 mice on Western blots of thymocyte total cell extracts probed with Sin-specific Ab. Two nonspecific bands (Non) are recognized by the Sin Ab on Western blots. The lower nonspecific band was included as a control for equal loading.

Effect of SinΔC expression on thymocyte development

Thymocyte development is a series of complex developmental events that culminates in the production of mature T cells from immature precursors. The different stages of thymocyte development are characterized by the sequential expression of surface markers such as CD44 and CD25, the pre-TCR, and the CD4 and CD8 coreceptors. $CD4^-CD8^-$ double-negative (DN) cells undergo rearrangement of the TCRβ and successful rearrangement together with pre-TCR engagement allows their progression to the $CD4^+CD8^+$ double-positive (DP) stage (37–41). $CD4^+CD8^+$ DP cells that have successfully rearranged their TCRα locus subsequently undergo positive or negative selection. Positively selected thymocytes down-regulate expression of either their CD4 or CD8 coreceptors to become mature $CD4^+$ or $CD8^+$ SP mature T cells (41, 42).

Initial analysis of transgenic animals revealed that the thymi of the SinΔC-expressing animals were smaller in size than the wild-type controls. Analysis of multiple animals showed that, consistent with the smaller thymus, the total transgenic thymocyte numbers recovered were ~40% of wild-type levels (Fig. 2*A*). The decrease in total number of thymocytes was due to significant decreases in the number of cells in the DP, CD4, and CD8 SP populations, and an observed, however, not significant reduction in the DN population, (Fig. 2*B*).

To further examine the effect of SinΔC expression on thymocyte development, thymocytes from wild-type and transgenic littermates were stained with fluorescently labeled mAbs against different surface molecules such as CD4, CD8, CD3ϵ, and CD69. A representative flow cytometric analysis of thymocytes from two founder lines, CR1 and MA2, is shown in Fig. 2*C*. In addition, the percentage of cells within different thymocyte populations in relation to total thymocytes is shown at the bottom of Fig. 2*C*. When equal numbers of thymocytes were stained, we found that the percentage of cells that were negative for CD4/CD8 expression (DN), as well as cells bearing both CD4 and CD8 coreceptors (DP thymocytes), were unaffected in the transgenic animals (Fig. 2*C*). However, the same phenotypic analysis of these thymocytes revealed a substantial decrease in the percentages of mature $CD4^+8^-$ and $CD4^-8^+$ SP cells (Fig. 2*C*). The $CD8^+$ population in particular was most severely affected as the proportion of these cells was decreased by ~70–90% as compared with normal controls (Fig. 2*C*). The effect of transgene expression on the percentage of $CD4^+$ SP cells was a reduction by ~50–60% (Fig. 2*C*). Taken together, these data suggest that SinΔC expression compromises the viability, but not the development of, DP thymocytes while specifically perturbing the maturation of SP cells.

To further characterize the maturation of thymocytes, we analyzed the expression of several thymocyte cell surface markers.

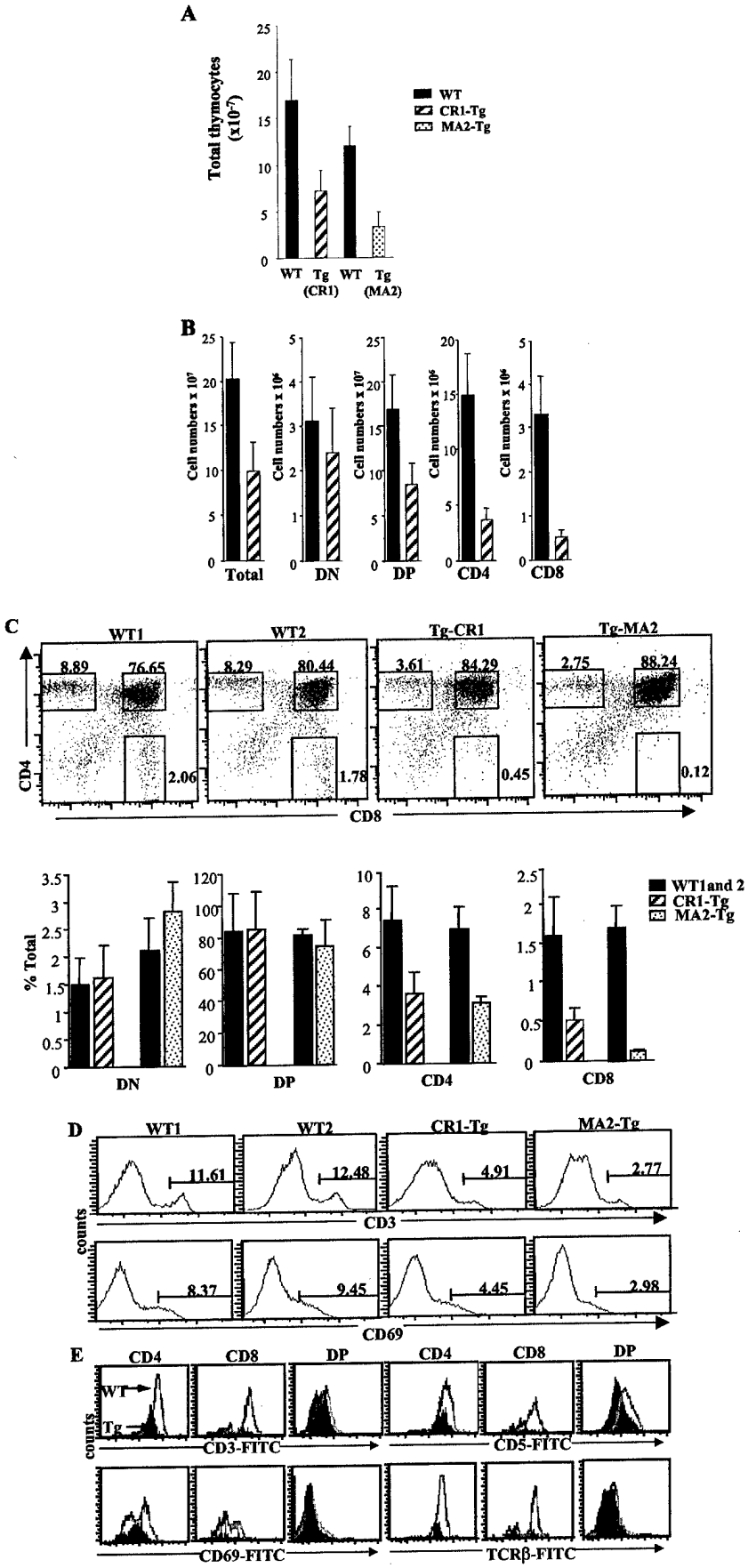

FIGURE 2. Expression of SinΔC in the thymus interferes with normal thymocyte maturation. *A*, Total thymocytes from 6- to 8-wk-old wild-type and transgenic animals were counted and averaged as shown. At least five animals from two different transgenic lines were used. *B*, Cells within each thymic subset were enumerated by FACS analysis. Results from at least 10 wild-type and 18 CR1 mice are represented as the mean ± SD. Similar results were obtained with the MA2 transgenic animals (data not shown). *C*, Thymocytes from 6- to 8-wk-old mice were stained with CD4-allophycocyanin and CD8-PerCP mAbs (1×10^6 cells/well) and analyzed by flow cytometry. Shown are CD4 vs CD8 plots of normal littermate controls and two transgenic lines CR1 and MA2 as shown. WT1 is litter-mate control for CR1 and WT2 for MA2. The numbers represent the percentage of thymocytes in the boxed areas. The bottom graphs represent percentages of cells within each population as shown, expressed in relation to total thymocyte numbers (mean ± SD). *D*, The histograms represent profiles of total thymocytes from wild type and transgenic animals stained with CD3 or CD69 Abs as shown. *E*, Histograms of DP, $CD4^+$ and $CD8^+$ cells stained with CD4/CD8 Abs and FITC-conjugated Ab specific for different surface markers as shown. Wild-type cell populations are represented by the gray line, transgenic cells by the filled curve.

The first, CD3ε, is a component of the TCR whose expression is up-regulated as thymocytes mature from the DP to the SP stage (43). The total $CD3^{high}$ mature T cell population normally represents ~15% of total thymocytes. Staining of SinΔC transgenic thymocytes revealed a 2- to 3-fold decrease in the total $CD3^{high}$ population (Fig. 2*D*, CD3, top histograms). Additionally, for total thymocytes we observed reduced expression levels of CD69 (Fig. 2*D*, bottom histograms), a maturation marker whose expression correlates with positively selected, maturing thymocytes (44). Decreased expression of the CD3 and CD69 markers in the total thymocyte population could be explained by the decrease in SP cell populations (Fig. 2*B*) or by abnormally low levels of expression in the SP population.

To decipher between these two possibilities, we further examined the role of SinΔC expression on thymocyte development by analyzing the levels of several cell surface markers on the individual DP and $CD4^+$ and $CD8^+$ SP cell populations. In addition to CD3ε and CD69, we also analyzed the expression levels of the β component of the TCR and CD5. CD5 is another maturation marker whose levels rise steadily during thymocyte maturation and is dependent on TCR signaling (45). Thus, thymocytes were triple-stained with CD4/CD8 and CD3ε, TCRαβ, CD5, or CD69 mAbs. The different thymocyte populations from wild-type and transgenic animals were defined on the basis of CD4/CD8 fluorescence intensity, and fluorescence histograms depicting CD3, CD5, CD69, or TCRβ expression on DP and SP cells are shown (Fig. 2*E*). The expression patterns for the different markers in all subpopulations were similar to the wild type, while the absolute numbers of cells in the $CD4^+$ and $CD8^+$ compartments were less in the transgenic animals (Fig. 2*E*). Thus, the reduction in CD3 and CD69 expression in the entire thymocyte population is solely due to the reduction of the $CD4^+$ and $CD8^+$ SP population, rather than lower levels of CD3/CD69 expression. Consistent with this, we also found that the decrease in T lymphocyte numbers was not confined to the thymus because the numbers of mature splenic T cells of both the CD4 and CD8 lineages were substantially reduced (~50%) in transgenic animals as compared with normal littermate controls (data not shown). In addition, staining of splenocytes with B cell-specific Abs (B220, IgM) revealed that the B cell compartment was intact, consistent with CD2-promoter-mediated, T cell-specific expression of SinΔC (data not shown). In summary, our results show that expression of SinΔC in thymocytes inhibits production of normal numbers of mature T cells and suggest that the truncated Sin mutant is a negative regulator of T lymphocyte differentiation.

Effect of SinΔC expression on thymocyte apoptosis

As shown above in Fig. 2*A*, the total transgenic thymocyte numbers recovered were ~40% of wild-type levels. DP thymocytes constitute the bulk of the thymus and we found that the actual cell numbers of transgenic DP thymocytes were reduced by 50% (Fig. 2*B*). However, the percentage of transgenic DP thymocytes recovered were similar to wild-type controls, suggesting that SinΔC expression affects the survival, but not the development, of these thymocytes. Thus, we examined whether the inhibitory effect of SinΔC was caused by enhanced levels of thymocyte apoptosis.

Freshly prepared thymocytes from wild-type and CR1 transgenic animals were cultured in medium for 6 h before staining with the vital dye 7AAD and annexin V. Typical results from two-parameter analyses are shown in Fig. 3*A* (dot plots). Indeed, we observed a higher percentage of annexin V-positive cells from transgenic animals (28.59%) as compared with wild type (9.60%),

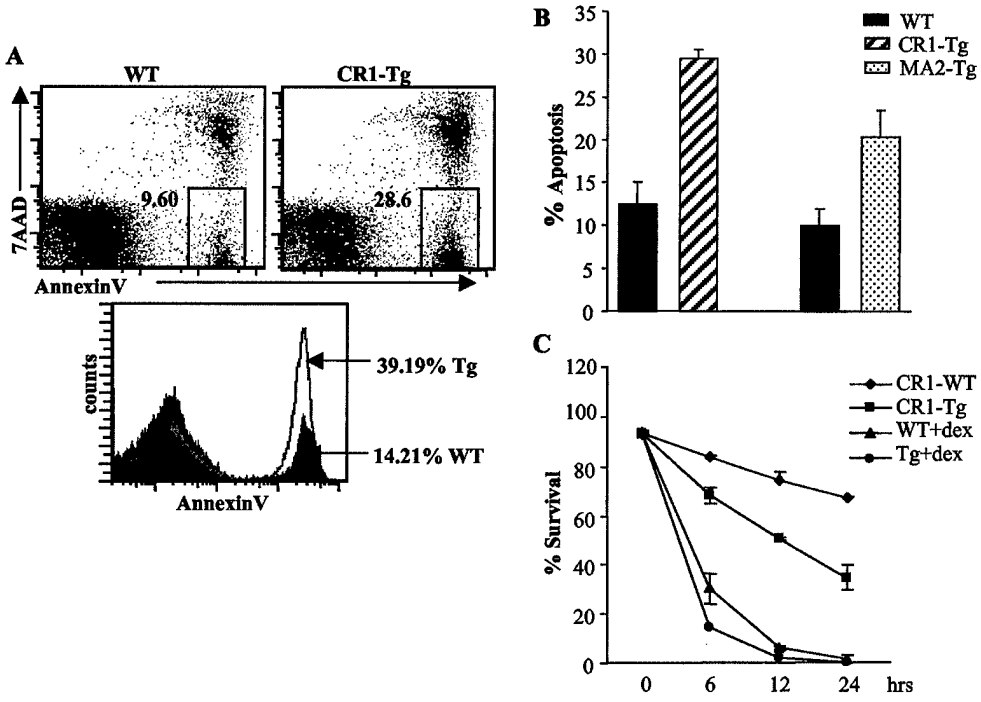

FIGURE 3. SinΔC expression increases thymocyte apoptosis. *A*, Total thymocytes from CR1 animals were cultured for 6 h and then stained with Annexin V^{PE} and 7AAD. Representative dot plots of a two-parameter FACS analysis are presented for wild-type and transgenic thymocytes. Boxed areas represent the percentages of cells undergoing early apoptosis (annexin V^+, $7AAD^-$). A histogram of total annexin V staining is shown at the bottom along with percentages of wild-type and transgenic cells undergoing cell death. *B*, Wild-type and transgenic thymocytes from CR1 and MA2 mice were harvested, cultured for 6 h, and then stained with 7AAD and annexin V. Percent apoptosis is represented as the number of cells that are annexin $V^+/7AAD^-$ (early apoptosis) averaged from three different experiments expressed as mean ± SD, for two transgenic founders as shown. *C*, Thymocytes from wild-type and transgenic CR1 mice were cultured with or without dexamethasone for the indicated times and then stained with 7AAD and annexin V. Percent survival is represented as the number of live cells (7AAD and annexin V-negative) remaining at each time interval.

thus revealing that there is enhanced spontaneous apoptosis in the transgenic thymocytes (Fig. 3*A*). This increase was consistent in cells undergoing early apoptosis (7AAD$^-$/annexin V$^+$) as well as in thymocytes at a later stage of cell death (7AAD$^+$/annexin V$^+$) (Fig. 3*A*, *right* and *bottom panels*). Results from several experiments using two different founder lines (CR1 and MA2) show a >2-fold increase in spontaneous thymocyte apoptosis in transgenic mice as compared with normal controls (Fig. 3*B*). We also found a concurrent decrease in transgenic thymocyte survival as determined by decreased numbers of live 7AAD$^-$/annexin V$^-$ cells over a 24-h period (Fig. 3*C*). Dexamethasone treatment of wild-type and transgenic cells was included in these experiments as a control for thymocyte apoptosis (Fig. 3*C*). These results suggest that expression of SinΔC reduces thymic cellularity by increasing thymocyte apoptosis, thus negatively regulating thymocyte survival.

Role of the Src kinases in SinΔC-mediated inhibition of thymocyte survival and maturation

Sin and similar adapter molecules exhibit no known enzymatic activity and modulate signaling through the formation of protein-protein interactions mediated by proline-rich and tyrosine-based motifs (29). Phosphorylation of the tyrosine-based motifs by tyrosine kinases is essential for binding of these residues to their ligands, which are SH2-domain-containing signaling intermediates (19, 33). In previous experiments, we found that the truncated form of Sin, used in this study as the transgene, can very efficiently bind to the Fyn- and Src-SH3 domains through its proline-containing motif, activate Src kinase signaling, and become constitutively phosphorylated on tyrosine residues (23, 29). We speculated that expression of the truncated mutant in thymocytes should then lead to Src kinase-mediated constitutive SinΔC phosphorylation and signaling. Given that Sin was cloned as a Src kinase ligand and Fyn and Lck are the most prevalent Src kinases in thymocytes, we tested whether Fyn and/or Lck could interact with SinΔC in transgenic thymocytes.

Thymocyte cell extracts from wild-type and transgenic animals were immunoprecipitated with Fyn- and Lck-specific Abs, and the immune complexes were incubated in the presence of radioactive ATP. We found that the SinΔC protein associated with and was phosphorylated by Fyn in vitro (Fig. 4*A*, *left panels*). In addition, we found that Fyn associated with phosphorylated Sin in anti-Sin immunoprecipitates (Fig. 4*A*, *right panels*). This was expected, given the specificity of the proline-rich motif of SinΔC for the Src and Fyn SH3 domains (23) which is a prerequisite for stable association of Sin with these kinases (16). In addition to SinΔC, we also tested the phosphorylation of the exogenous substrate, enolase, which has traditionally been used to assay for increased Src kinase activity (35). As with SinΔC, enolase was also phosphorylated in transgenic, but not wild-type, cell extracts, consistent with the proposed SinΔC-induced activation of Fyn (Fig. 4*B*). In contrast to Fyn, we did not observe SinΔC binding to Lck (Fig. 4*A*, *middle panels*). This observation is not surprising given that the proline-rich motifs of Sin do not recognize the Lck-SH3 domain (16). This is due to the fact that the Lck SH3 domain is the most divergent within the Src family, recognizing a different consensus sequence than the Fyn and Src SH3 domains which is not present on Sin (23, 46, 47). Thus, these results show that SinΔC preferentially binds to Fyn in thymocytes and suggest that Fyn-dependent phosphorylation of SinΔC may be important for the inhibitory effects of SinΔC in thymocyte development.

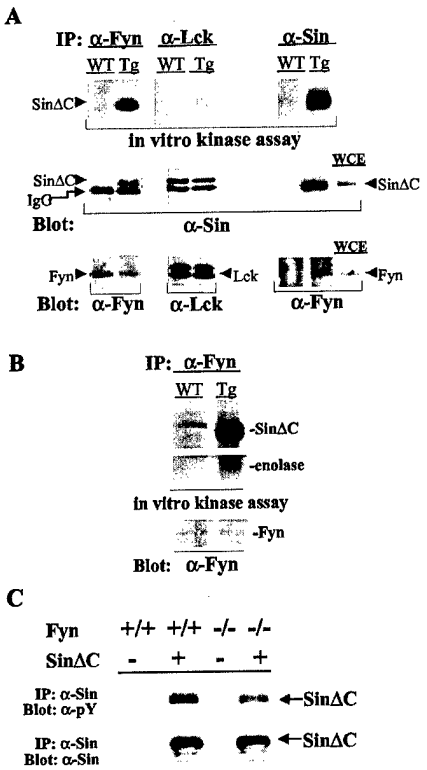

FIGURE 4. SinΔC interacts with the Src kinase Fyn in thymocytes. Thymocyte cell extracts from wild-type and SinΔC transgenic mice were immunoprecipitated with Fyn-, Lck- and Sin-specific Abs, and immune complexes were incubated in vitro with [γ^{32}-P]ATP for 10 min. Proteins were separated by electrophoresis and blotted onto nitrocellulose membrane. The membrane was exposed on film for 24 h at −70°C (*top panels*). Then the same membrane was first incubated with Sin-specific Ab (*middle panels*), and then were stripped and reprobed with either Fyn- or Lck-specific Abs as shown (*bottom panels*). Protein bands were visualized by ECL. In the *middle panel*, the bands from the Lck immunoprecipitation probed with the Sin Ab are nonspecific bands. *B*, Thymocyte cell extracts were immunoprecipitated and kinase assays performed as in *A*, except that 5 μg of purified enolase were exogenously added to each tube. The membrane was stripped and reprobed with Fyn-specific Ab. *C*, SinΔC-expressing thymocytes from wild-type or *fyn*$^{-/-}$ animals were immunoprecipitated with anti-Sin Ab and Western blotted first with antiphosphotyrosine and then with Sin-specific Abs. Protein bands were visualized by ECL.

Fyn is required for SinΔC-mediated thymocyte apoptosis but not for SinΔC-mediated inhibition of thymocyte maturation

Given that SinΔC preferentially binds to and is phosphorylated by Fyn in thymocytes (Fig. 4*A*), we speculated that Fyn-mediated phosphorylation of SinΔC in the thymus may regulate the inhibitory effects of SinΔC on thymocyte survival and maturation. To test the role of Fyn in SinΔC-mediated inhibition of thymocyte maturation and survival, we crossed the SinΔC-expressing mice to *fyn*$^{-/-}$ animals (31, 32). Thymocytes from normal and SinΔC-expressing animals in a *fyn*$^{+/-}$ or *fyn*$^{-/-}$ background were stained with CD4/CD8 and analyzed by flow cytometry. We found that in the absence of Fyn, SinΔC still blocked thymocyte maturation, again shown by reduced percentages of CD4$^+$, and especially CD8$^+$, SP cells (Fig. 5*A*). Thus, these data suggest that Fyn is not required for the inhibitory effect of SinΔC in thymocyte differentiation. Fyn$^{-/-}$/SinΔC$^-$ thymocytes were also analyzed and found to be similar to Fyn$^{+/-}$/SinΔC$^-$ thymocytes (not shown), consistent with data obtained with *fyn*$^{-/-}$ animals showing that the absence of Fyn has no effect on thymocyte development (31, 32). In

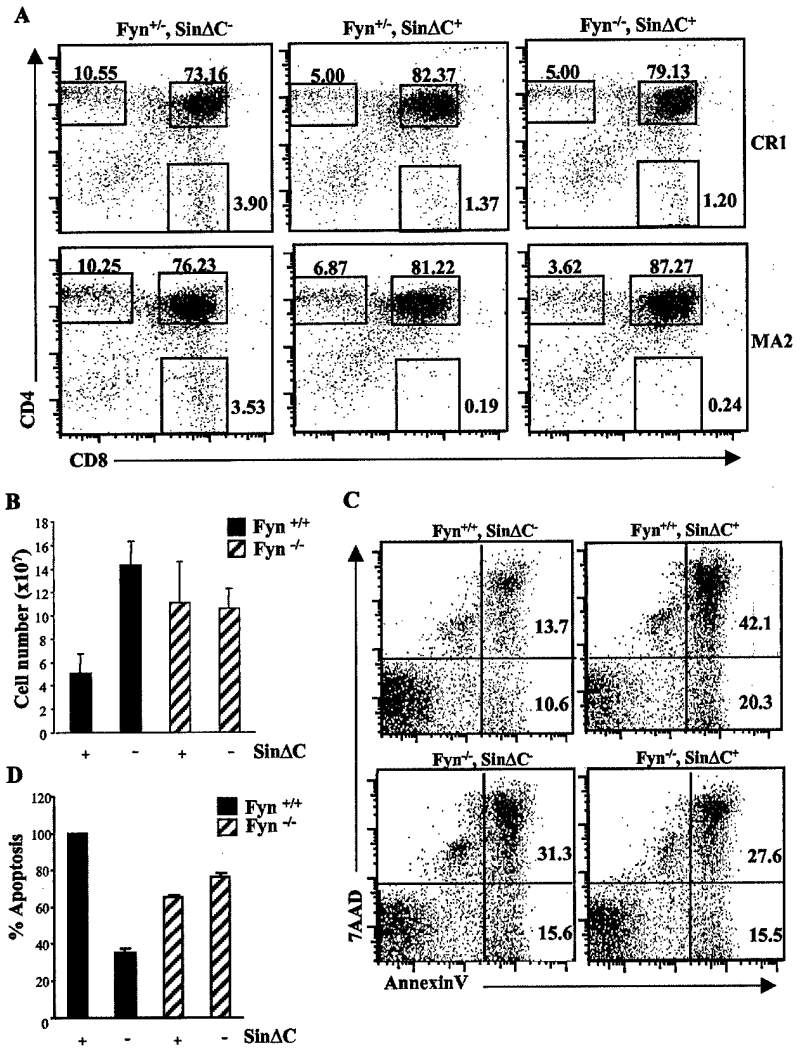

FIGURE 5. Fyn is dispensable for the inhibitory effect of SinΔC on thymocyte maturation but is required for SinΔC-mediated thymocyte apoptosis. *A*, Thymocytes from 6- to 8-wk-old mice were stained with anti-CD4 and anti-CD8 Abs and analyzed by flow cytometry as in Fig. 2. Shown are CD4 vs CD8 plots of normal or transgenic mice from two transgenic lines (CR1, MA2) in heterozygous or null *fyn* background as shown. The numbers represent the percentages of thymocytes in the boxed areas. A representative analysis of four experiments is shown. *B*, Freshly isolated thymocytes were counted and total cell numbers were plotted as shown expressed as mean ± SD ($n = 5$). *C*, Cells from wild-type or $fyn^{-/-}$ animals in the presence or absence of SinΔC were also incubated at 37°C for 6 h, stained with annexin V and 7AAD and analyzed by flow cytometry. *D*, Percent apoptosis is represented as the percentage of cells that are annexin $V^+/7AAD^-$ (early apoptosis) from different samples, normalized to percent apoptosis obtained from staining $Fyn^{+/+}/SinΔC^+$ thymocytes which was given an arbitrary value of 100.

addition, we found that although SinΔC is a substrate of, and is phosphorylated by Fyn in vitro (Fig. 4*A*), immunoprecipitation of SinΔC from $fyn^{-/-}/SinΔC^+$ thymocytes shows reduced but significant residual SinΔC phosphorylation (Fig. 4*C*). These results suggest that in addition to Fyn, other kinases phosphorylate SinΔC and may regulate the effects of this protein on thymocyte maturation.

Subsequently, we examined whether the effect of SinΔC-mediated reduction in total thymocyte numbers required Fyn. Consistent with the results shown above (Fig. 2*A*) we found a substantial reduction in total thymocyte numbers in cells expressing SinΔC in a wild-type Fyn background as compared with normal thymocytes (Fig. 5*B*). However, in the absence of Fyn, SinΔC expression had no effect on thymocyte numbers, suggesting that Fyn is required for the decrease in thymic cellularity observed in SinΔC transgenic animals (Fig. 5*B*). To further address the role of Fyn in SinΔC-mediated thymic atrophy, we examined thymocyte apoptosis in a $SinΔC/fyn^{-/-}$ background. To this end, thymocytes from wild-type and transgenic animals in a wild-type or null *fyn* background were analyzed as in Fig. 3. Annexin V and 7AAD staining revealed that SinΔC expression led to increased spontaneous thymocyte apoptosis (Fig. 5*C*) consistent with the results presented in Fig. 3. In contrast, although we observed higher levels of apoptosis in $fyn^{-/-}$ thymocytes, no further effect was observed in the presence of SinΔC (Fig. 5, *C* and *D*), again suggesting that Fyn is required for the effects of SinΔC on thymocyte apoptosis. Similar results were obtained with thymocytes from MA2 transgenic animals (not shown).

SinΔC expression does not inhibit phosphorylation events proximal to the TCR

Given that Fyn regulates phosphorylation events proximal to the TCR after receptor cross-linking (31, 32), we examined whether SinΔC expression had an effect on total protein tyrosine phosphorylation induced in response to TCR cross-linking. Thymocytes from wild type and SinΔC animals were stimulated for the indicated times by TCR cross-linking using anti-CD3ε-specific Ab and Western blots of total cell lysates were immunoblotted with antiphosphotyrosine-specific Ab. We found no discernible differences in the pattern of tyrosine phosphorylated proteins between wild type and transgenic lysates with the notable exception of SinΔC, which is prominently and constitutively phosphorylated in transgenic cell lysates (Fig. 6*A*). This observation was consistent with previous experiments (29) and our rationale that the truncated mutant would efficiently interact with Src kinases and be effectively phosphorylated. Thus, our results suggest that SinΔC expression has no effect on Lck/Fyn-mediated phosphorylation events proximal to the TCR and that SinΔC may exert its inhibitory effects downstream of the receptor and the signal-initiating phosphorylation events. These results also suggest that SinΔC is

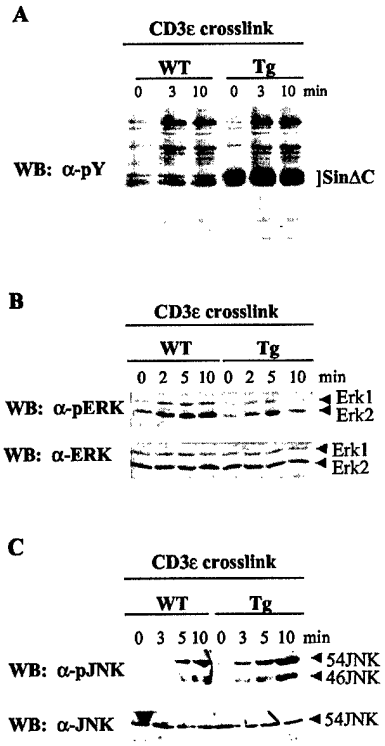

FIGURE 6. Normal total tyrosine phosphorylation but reduced ERK activation in SinΔC-expressing thymocytes. Thymocytes were cross-linked with anti-CD3 Ab for the indicated times at 37°C. Western blots of total cell extracts were incubated with the following Abs: anti-phosphotyrosine (*A*), anti-phospho-ERK and anti-phospho-JNK (*B* and *C*, *top panels*), stripped and reprobed with anti-ERK or anti-JNK as shown (*B* and *C*, *bottom panels*). Protein bands were visualized using ECL.

not acting as a competitive inhibitor for Fyn/Lck-mediated substrate phosphorylation due to overexpression.

SinΔC expression inhibits ERK, but not JNK, MAPK phosphorylation

Given the lack of an effect of SinΔC expression on tyrosine phosphorylation events proximal to the TCR, we examined the effect of SinΔC on downstream signaling events such as phosphorylation of the MAPKs ERK and JNK. Activation of these kinases in thymocytes is the result of TCR-mediated activation of the Ras signaling cascade (48, 49). Total thymocytes from normal or SinΔC mice were incubated with anti CD3ε Ab for different times at 37°C. Total cell extracts were fractionated and blotted with anti-phospho-ERK or JNK Abs which recognize the phosphorylated, active forms of ERK1,2 and JNK. In normal thymocyte cell extracts, phosphorylation of ERK1,2 was rapidly stimulated in response to TCR cross-linking, and was sustained for at least 10 min (Fig. 6*B*, *top panel*). In contrast, ERK1,2 phosphorylation in thymocyte cell extracts from SinΔC mice was less pronounced and was rapidly down-regulated (Fig. 6*B*, *top panel*). In contrast, JNK phosphorylation was intact in transgenic lysates immunoblotted with phospho-JNK-specific Ab (Fig. 6*C*, *top panel*). The levels of total ERK1,2 and JNK proteins, revealed by immunoblotting with specific Abs against total ERK and JNK, were similar in all cases (Fig. 6, *B* and *C*, *bottom panels*). These data show that the levels as well as the kinetics of ERK1,2 stimulation in SinΔC thymocytes are inhibited as compared with normal controls and suggest that SinΔC may inhibit thymocyte function by interfering with normal ERK activation.

Discussion

In this report, we examined the role of the novel adapter molecule Sin in T lymphocyte function. We used this system for our studies because the thymus is one of the tissues in which Sin is most highly expressed. In addition, two Src kinases, Lck and Fyn, potentially involved in Sin phosphorylation, are also expressed in the thymus and are important for T lymphocyte function. In previous studies, we showed that full-length Sin is less potent in its ability to mediate Src kinase signaling as compared with a truncated form, SinΔC. Therefore, in the present study we expressed SinΔC in the thymocytes of transgenic animals with the assumption that truncated Sin would act as an activated mutant that would more efficiently interact with and become phosphorylated by endogenous Src kinases. Efficient phosphorylation of Sin by Src and possibly other kinases is essential for the adapter function of Sin. Consistent with this, SinΔC was constitutively phosphorylated by Fyn in transgenic thymocytes. We found that expression of SinΔC inhibited thymocyte maturation, shown by reduced percentages of $CD4^+$ and particularly $CD8^+$ cells, and induced thymocyte apoptosis. We also found that Fyn was required for the effect of SinΔC on thymocyte apoptosis but not for inhibition of thymocyte transition to SP cells. In the same experiments, SinΔC failed to associate with and become phosphorylated by Lck.

The observation that Fyn is not required for the negative effects of SinΔC on mature T cell production is not surprising given that $fyn^{-/-}$ animals exhibit normal T cell maturation (31, 32). Therefore, SinΔC may be exerting this inhibitory effect through association with other kinases and/or intracellular signaling molecules. Consistent with the involvement of kinase(s) other than Fyn or Lck is our observation that there is significant residual tyrosine phosphorylation of SinΔC in $fyn^{-/-}$ thymocytes. Although the identity of this kinase(s) is currently unknown, efforts are under way to identify additional proteins that may phosphorylate SinΔC on tyrosine residues. Alternatively, other conserved domains mediating protein-protein interactions such as proline-rich motifs may be mediating the effects of Sin.

Although Fyn is not required for SinΔC-mediated inhibition of thymocyte maturation, Fyn is necessary for the effects of SinΔC on thymic cellularity and thymocyte apoptosis. Indeed, we found that in the absence of Fyn, SinΔC had no effect on thymocyte numbers and thymocyte apoptosis (Fig. 5, *B* and *C*). We also observed a consistent reduction of total thymocyte numbers in $fyn^{-/-}$ animals and increased apoptosis as compared with wild-type cells (Fig. 5, *B* and *C*). This finding could be explained by existing evidence suggesting that Fyn contributes to the generation of DP thymocytes. In *lck* null animals, the production of mature SP T cells is severely impaired whereas DP thymocytes are being produced albeit at reduced levels (30). However, the presence of these DP cells is completely obliterated in the $fyn^{-/-}/lck^{-/-}$ double null background, suggesting that Fyn can compensate for Lck in the production of DP cells (50, 51). This could explain the reduction in thymocyte numbers and increased apoptosis we observe with $fyn^{-/-}$/SinΔC animals. We found that SinΔC expression causes no additional decrease on thymocyte numbers in the absence of Fyn, suggesting that SinΔC-induced apoptosis requires Fyn. Thus, the increase in thymocyte apoptosis in transgenic animals could be the result of either a novel inhibitory signal mediated by phosphorylated SinΔC or of dominant inhibition of Fyn function by overexpressed SinΔC.

In our experiments, we found that SinΔC is prominently phosphorylated in unstimulated transgenic cells, and that there may be

a small increase in its phosphorylation in response to TCR cross-linking (Fig. 6*A*). More importantly, there are no apparent differences in the total protein tyrosine phosphorylation in the transgenic cells as compared with wild-type controls, in cell extracts from stimulated or unstimulated cells (Fig. 6*A*), unlike the reduced phosphorylation of substrates observed in *fyn* null thymocytes (31). The lack of a positive or negative effect of SinΔC expression on tyrosine phosphorylation has two implications. First, data presented in Fig. 4 suggest that expression of SinΔC in thymocytes leads to Fyn activation and SinΔC phosphorylation. If Fyn is indeed activated, we should observe increased substrate phosphorylation and a phenotype similar to that of mice overexpressing Fyn, i.e., hyperstimulatable thymocytes and increased substrate phosphorylation (52). This is opposite to our results with SinΔC-activated Fyn. We believe this is due to the fact that ligand-activated Src kinases behave differently than their constitutively active counterparts that are induced as a result of mutations or overexpression. Thus, in contrast to constitutively activated Src kinases, expression of SinΔC with Src kinase in a cell culture system does not lead to increased substrate phosphorylation and the signaling mechanism of SinΔC-activated Src is different from signaling through constitutively active Src (16, 29).

Second, the lack of a negative effect of SinΔC expression on substrate phosphorylation argues against a dominant inhibitory effect for SinΔC on Fyn- and/or Lck-mediated phosphorylation due to SinΔC overexpression and supports the existence of a novel inhibitory signal(s) mediated by SinΔC. Consistent with this, we found that phosphorylation of the MAPK ERK was impaired in SinΔC-expressing cells as compared with normal controls. This effect was specific to ERK because phosphorylation of the related MAPK JNK was normal. These results suggest that SinΔC may be specifically inhibiting ERK activation through a mechanism downstream of the phosphorylation events proximal to the TCR.

During thymocyte maturation, $\alpha\beta$ T cells undergo positive or negative selection, events that are regulated by TCR ligation of self-peptide-MHC complexes on epithelial cells of the cortex. There is substantial evidence that ERK kinase is an important regulator of positive selection of thymocytes. Early experiments with dominant negative inhibitors of the Ras-MAP or ERK kinase pathway showed that ERK activation through the Ras signaling cascade is required for positive selection and thymocyte maturation whereas negative selection proceeds unimpaired (53, 54). More recent evidence has shown that ERK activation can regulate both selection processes in immature thymocytes (55, 56). The threshold model can explain these apparently conflicting results. This model suggests that the kinetics and extent of ERK activation in response to TCR ligation determine positive vs negative selection (57–59). Collectively, these data suggest that ERK activation is an important signaling event in thymocyte maturation.

In our system, we found that ERK activation is impaired in thymocytes expressing SinΔC (Fig. 6*B*). The effect of SinΔC on thymocyte maturation is strikingly similar to the effects observed in p44 MAPK (ERK1)-deficient mice. In these mice, thymocyte maturation beyond the DP $CD4^+CD8^+$ stage is reduced by half, with a similar decrease in thymocytes expressing high levels of the TCR ($CD3^{high}$) (60). These same phenotypic features are present in the SinΔC transgenic thymocytes. It is thus possible that reduced ERK activation and, as a consequence, alteration of signaling thresholds is responsible for the effects of SinΔC on thymocyte maturation in our system. SinΔC-mediated changes in TCR-dependent signaling thresholds could in turn affect the positive and/or negative selection of SinΔC-expressing DP thymocytes. Efforts are currently under way to elucidate the molecular mechanisms of SinΔC-mediated inhibition of ERK phosphorylation and its effects on positive and negative selection. To this end, SinΔC mice that have been sufficiently backcrossed to the C57/B6 background will be crossed to TCR transgenic mice, such as the H-Y TCR transgenic animals, to address the role of SinΔC expression on positive and/or negative selection.

Finally, because in our experiments we are using an activated form of Sin, SinΔC, we believe that the effects of the truncated mutant on thymocyte maturation and apoptosis reflect the function of endogenous Sin and that endogenous Sin is a negative regulator of T lymphocyte function. This is supported by experiments in Jurkat cells showing that both full-length Sin and SinΔC inhibit activation of NFAT and AP-1 reporter constructs in response to TCR stimulation and act as negative regulators of T cell activation (our unpublished observations). Experiments to further explore these observations are currently under way in the form of generating transgenic mice expressing full-length Sin and Sin knockout animals. We anticipate that these experiments will support a role for Sin as a negative regulator of T lymphocyte function and will provide novel insight into TCR-mediated signaling pathways.

Acknowledgments

We thank Jerry Siu and Yong-Rui Zou for critically reading the manuscript and Michael Weston for his input and helpful discussions.

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