Chk, a Csk Family Tyrosine Protein Kinase, Exhibits Csk-like Activity in Fibroblasts, but Not in an Antigen-specific T-cell Line*

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The Csk family of tyrosine protein kinases comprises two members named Csk and Chk. These enzymes phosphorylate the carboxyl-terminal tyrosine of Src-related kinases in vitro, thereby repressing their activity. Csk has been found to be necessary for normal embryonic development, and to be a potent negative regulator of antigen receptor signaling in T-lymphocytes. As the Csk family genes are expressed in all cell types (1; reviewed in Refs. 2 and 3). As is the case for members of the Src family, Csk contains amino-terminal Src homology (SH)³ and SH2 domains, as well as a carboxyl-terminal catalytic domain. In contrast to Src-related enzymes, however, p50⁴–⁵ does not possess an amino-terminal site of myristoylation, a site of autophosphorylation, or a carboxyl-terminal site of tyrosine phosphorylation (Fig. 1). Significant interest in p50⁴–⁵ stems from its ability to phosphorylate the carboxyl-terminal tyrosine of Src family kinases in vitro, thereby repressing their enzymatic activity.

Csk is a 50-kilodalton (kDa)-cytosolic tyrosine protein kinase expressed in all cell types (1; reviewed in Refs. 2 and 3). As is the case for members of the Src family, Csk contains amino-terminal Src homology (SH)³ and SH2 domains, as well as a carboxyl-terminal catalytic domain. In contrast to Src-related enzymes, however, p50⁴–⁵ does not possess an amino-terminal site of myristoylation, a site of autophosphorylation, or a carboxyl-terminal site of tyrosine phosphorylation (Fig. 1). Significant interest in p50⁴–⁵ stems from its ability to phosphorylate the carboxyl-terminal tyrosine of Src family kinases in vitro, thereby repressing their enzymatic activity.

The importance of Csk in normal cellular physiology was first highlighted by the observation that Csk-deficient mice, generated by homologous recombination in embryonic stem cells, exhibited severe abnormalities in the central nervous system and early embryonic lethality (4, 5). Furthermore, Csk-deficient embryonic stem cells were unable to differentiate into T- or B-cells, when injected into blastocysts (6). Support for the notion that Csk functions in vivo by repressing Src family kinases was lent by the observation that fibroblasts derived from Csk-deficient embryos possessed hyperactive Src family kinases (p60²–⁵, p59⁴–⁵, and p56⁴–⁵) and elevated levels of phosphotyrosine (4, 5). p50⁴–⁵ also plays several critical roles in mature cellular physiology (7–10). Notably, we reported that overexpression of Csk in an antigen-specific T-cell line (BI-141) caused a pronounced inhibition of T-cell receptor (TCR)-induced tyrosine protein phosphorylation and lymphokine secretion (7, 8).

Recently, several groups identified a second member of the Csk family (11–17). This enzyme, variably named Ntk, Matk, Chk, Hyl, Lsk, and Batk, is now termed Chk (for Csk homologous kinase). We and others (11, 12, 18) have shown that Chk can phosphorylate the inhibitory carboxyl-terminal tyrosine of several Src-related enzymes in vitro, including Lck, Fyn, and c-Src. Unlike Csk, Chk only accumulates in brain and hemopoietic cells. Moreover, as a consequence of alternative splicing, the chk gene codes for two distinct proteins, p52⁴–⁵ and p56⁴–⁵, which differ by the absence or presence of a 40-amino acid extension at their amino terminus (19; Fig. 1). Although the purpose of these two different Chk polypeptides is not determined, p52⁴–⁵ primarily abounds in brain, whereas p56⁴–⁵ is predominantly contained in hemopoietic cells. However, it should be pointed out that, while p56⁴–⁵ is prominently expressed in human hemopoietic cells (15), it is generally a minor component of Chk proteins in mouse hemopoietic cells (19). The significance of this species difference is not understood.

Little is known of the role(s) of Chk in normal cellular physiology. Because it is seemingly always expressed with Csk, it is reasonable to speculate that the two enzymes may not serve fully identical functions. To begin dissecting these roles, we have tested the capacity of Chk to execute p50⁴–⁵-type functions in vivo. Our studies showed that, like Csk, Chk was apt at reducing the abundance of phosphotyrosine-containing proteins and the increased activity of p60²–⁵ and p59⁴–⁵ in Csk-deficient mouse embryo fibroblasts (MEFs). Unlike Csk, however, Chk was inefficient at repressing antigen receptor-induced signal transduction in T-cells. While these results supported the notion that Chk is also a negative regulator of Src family kinases in vivo, they implied that Chk may have a restricted Csk-like biological activity in mammalian cells.

MATERIALS AND METHODS

Cells—Csk-deficient and wild-type MEFs immortalized with simian virus (SV) 40 large T antigen were established by Imamoto and Soriano...
and provided by Drs. Brian Howell and Jon Cooper (Fred Hutchinson Cancer Center, Seattle, WA). NIH 3T3 fibroblasts expressing a chimera bearing the SH3 and SH2 regions of Csk and the kinase domain of Chk (Chk-Chk chimera) will be described elsewhere.2 BI-141 is an antigen-specific mouse T-cell hybridoma specific for the antigen bee venom. It was generated by retrovirus-mediated gene transfer of the cDNAs encoding mouse p52

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protein A (Amersham Canada, Oakville, Ontario, Canada) or 125I-antibody (Amersham Canada Ltd., Montréal, Quebec, Canada), immunoreactive products were detected by autoradiography and quantitated with a PhosphorImager.

Immune Complex Kinase Reactions—These experiments were done under linear conditions (Fig. 2, top panel) and were performed as described previously (33). They were conducted in the presence of acid-denatured rabbit muscle enolase as a model substrate. Data were quantitated with a PhosphorImager.

Cell Fractionation—For cell fractionation, cells were incubated for 15 min in hypotonic buffer (10 mM Tris, pH 7.4, 2 mM EDTA pH 8.0) supplemented with the protease and phosphatase inhibitors outlined above. Membranes were mechanically broken using a Dounce homogenizer. In all cases, staining with trypan blue confirmed that over 95% of cells had been lysed (data not shown). After adjusting the homogenates to 0.15 M NaCl, intact cells, nuclei, and large membrane sheets were removed by two successive centrifugations at 450 × g for 5 min. Supernatants were then separated into soluble (S100) and particulate (P100) fractions by ultracentrifugation at 100,000 × g for 30 min. The various fractions were extracted in boiling sample buffer. Partially

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tinin, phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (100 mM), and p38 mitogen-activated protein kinase (MAPK) inhibitors p38 MAPK inhibitors SB203580 (SB) and SP600125 (Sp). Immunoblots were performed according to a previously described protocol (32). After incubation with

Toxicologists, Lexington, KY. Anti-TCR MAb F23.1 was described elsewhere (3). Antibody-mediated T-cell Activation—T-cells were activated by stimulation for 2 min at 37 °C with anti-TCR Vβ8 mouse MAB F23.1 and short-chain mouse IgG, as outlined elsewhere (3). After activation, cells were lysed in boiling sample buffer, and lysates were processed for anti-phosphotyrosine immunoblotting. To measure lymphokine secretion, cells were incubated for 24 h with various concentrations of MAB F23.1 coated on plastic, according to a protocol detailed elsewhere (34). Release of interleukin-2 (IL-2) was determined by testing the ability of serial dilutions of the supernatants to support growth of the IL-2-dependent T-cell line HT-2. Units of IL-2 were calculated using a titration curve generated with recombinant IL-2 as a reference.

Antigen-induced T-cell Activation—Antigen stimulation assays were conducted by incubating BI-141 derivatives with irradiated spleen cells expressing AαAβ class II MHC molecules (obtained from a cross between B10 and B10.BR mice; The Jackson Laboratories, Bar Harbor, ME) and pulsed with various concentrations of beef insulin. After 24 h of stimulation with 100 μCi/ml Translabel (ICN Pharmaceuticals Canada Ltd., Montréal, Quebec, Canada) in methionine-free RPMI 1640 medium containing 2% dyes were filtered through a nitrocellulose filter and were assayed for lymphokine release by measuring their ability to support [3H]thymidine incorporation into HT-2 cells. Controls were without addition.

RESULTS

Retrovirus-mediated Transfer of chk cDNAs in Csk-deficient Mouse Embryo Fibroblasts—To test whether Chk can mediate Csk-type functions in mammalian cells, we examined whether it could restore the regulation of tyrosine protein phosphorylation in Csk-deficient MEFs (4, 5, 35). Cells were infected with retroviruses encoding the puromycin resistance gene (puro) alone or in combination with p52

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chimeric mouse model for the regulation of cell growth and differentiation (Fig. 1). Our work demonstrates that Chk functions as an important mediator of Csk-type functions in mammalian cells. chimeric mouse model for the regulation of cell growth and differentiation (Fig. 1). Our work demonstrates that Chk functions as an important mediator of Csk-type functions in mammalian cells.
control MEFs (lanes 1 and 2) which are devoid of endogenous Chk, cells infected with retroviruses encoding p52\(^{chk}\) (lanes 5 and 6) or p56\(^{chk}\) (lanes 7 and 8) contained appreciable quantities of the relevant Chk protein. In agreement with our earlier report (19), the expression levels of p52\(^{chk}\) were consistently greater than those of p56\(^{chk}\) in these cells. The basis for this difference is not known.

To ensure that equivalent amounts of Chk and Csk were expressed in these cell lines, we took advantage of NIH 3T3 cells expressing a chimeric protein bearing the SH3 and SH2 domains of Csk and the catalytic region of Chk (Csk-Chk chimera). This polypeptide reacted both with antibodies directed against the SH3 domain of p50\(^{chk}\) (Fig. 2B, top panel, lanes 6–8) and with antibodies against the kinase domain of Chk (bottom panel, lanes 7–9). Hence, using Csk-Chk as an internal standard, we could show that the range of expression of either p52\(^{chk}\) or p56\(^{chk}\) was roughly equivalent to that of p50\(^{chk}\) (compare top panel, lanes 3 and 4 with bottom panel, lanes 2–5).

**Regulation of Phosphotyrosine Levels by Chk in Csk-deficient Cells**—The abundance of phosphotyrosine-containing proteins in these cells was examined by anti-phosphotyrosine immunoblotting of total cell lysates (Fig. 3). Consistent with earlier reports (4, 5, 35, 36), MEFs lacking p50\(^{chk}\) (lane 2) contained markedly elevated levels of phosphotyrosine, in comparison with either MEFs derived from wild-type mice (lane 1) or Csk\(^{-/-}\) MEFs expressing p50\(^{chk}\) (lanes 3 and 4). Interestingly, Csk-deficient MEFs containing either p52\(^{chk}\) (lanes 5 and 6) or p56\(^{chk}\) (lanes 7 and 8) exhibited a striking reduction in tyrosine-phosphorylated proteins, in comparison with MEFs devoid of p50\(^{chk}\) (lane 2). Such a diminution affected all appreciable substrates. However, it is noteworthy that this decrease was slightly less than that observed in cells expressing p50\(^{chk}\) (compare lanes 5–8 with lanes 1, 3, and 4).

It has been demonstrated that components linked to the cytoskeleton, such as cortactin and paxillin, contain elevated amounts of phosphotyrosine in Csk-deficient MEFs (35, 36). To directly ascertain whether expression of Chk regulated these products, they were immunoprecipitated with specific antibodies and immunoblotted with anti-phosphotyrosine antibodies (Fig. 4, A and B, top panel). The abundance of these proteins was also monitored by immunoblotting of parallel immunoprecipitates with anti-cortactin or anti-paxillin antibodies, respectively (bottom panel). These analyses revealed that p52\(^{chk}\) and p56\(^{chk}\) markedly reduced the phosphotyrosine content of the 80-kDa cortactin (Fig. 4A) and 68–74-kDa paxillin (Fig. 4B).

Similar observations were made for p125\(^{ckb}\) and AFAP-110, two other components of the cytoskeleton (data not shown).

The differential ability of Csk and Chk to regulate tyrosine phosphorylation of predominantly cytosolic substrates was also

**Fig. 1. Primary structure of Csk family tyrosine protein kinases**. Schematic representations of the primary structure of the three Csk-related tyrosine protein kinases as well as of membrane-targeted derivatives (Src-Csk and Src-Chk).

**Fig. 2. Expression of p52\(^{chk}\) and p56\(^{chk}\) in Csk-deficient mouse embryo fibroblasts**. p52\(^{chk}\), p56\(^{chk}\), or p50\(^{chk}\) was expressed in Csk-deficient MEFs by retrovirus-mediated gene transfer. Monoclonal puromycin-resistant cell lines were established by limiting dilution. A, anti-Csk and anti-Chk immunoblots. Levels of Csk and Chk in representative cell lines were determined by immunoblotting of total cell lysates with antisera directed against the carboxyl-terminal tail of Csk (top panel) or the kinase region of Chk (bottom panel). Lane 1, wild-type MEFs (csk\(^{-/-}\)); lane 2, Csk-deficient MEFs (csk\(^{-/-}\)) + puro gene alone; lanes 3 and 4, csk\(^{-/-}\) MEFs + p50\(^{chk}\) (clones 7 and 10, respectively); lanes 5 and 6, csk\(^{-/-}\) MEFs + p52\(^{chk}\) (clones 5 and 10, respectively); and lanes 7 and 8, csk\(^{-/-}\) MEFs + p56\(^{chk}\) (clones 2 and 7, respectively). The positions of Csk, p52\(^{chk}\), and p56\(^{chk}\) are shown on the left, whereas those of precipitated molecular mass markers in kilodaltons are indicated on the right. Exposures: top panel, 18 h; bottom panel, 4 h. B, titration of levels of expression of Csk and Chk. The relative abundance of Csk and Chk in MEF derivatives was determined by comparison with serial dilutions of a Csk-Chk standard expressed in NIH 3T3 cells. Lysates were immunoblotted either with an antiserum reacting against the SH3 region of Csk (top panel) or with an antiserum directed against the kinase domain of Chk (bottom panel). Top panel: lane 1, csk\(^{+/+}\) MEFs; lane 2, csk\(^{-/-}\) MEFs; lanes 3 and 4, csk\(^{-/-}\) MEFs + p50\(^{chk}\) (clones 7 and 10, respectively). Bottom panel: lane 1, csk\(^{-/-}\) MEFs; lanes 2 and 3, csk\(^{-/-}\) MEFs + p52\(^{chk}\) (clones 5 and 10, respectively); lanes 4 and 5, csk\(^{-/-}\) MEFs + p56\(^{chk}\) (clones 2 and 7, respectively). Lane 5 (top panel) and lane 6 (bottom panel), neomycin-resistant NIH 3T3 cells (Neo); and lanes 6–9 (top panel) and lanes 7–9 (bottom panel), serial dilutions of lysates from Csk-Chk-expressing NIH 3T3 cells. Based on this titration, it was estimated that the relative amounts of Csk or Chk in these various cell lines were (considering the abundance of Csk in csk\(^{+/+}\) MEFs as 1.0) as follows: csk\(^{-/-}\) MEFs expressing p50\(^{chk}\); clone 7: 1.5 and clone 10: 7.5; csk\(^{-/-}\) MEFs expressing p52\(^{chk}\); clone 5: 4.5 and clone 10: 7.5; and csk\(^{-/-}\) MEFs expressing p56\(^{chk}\); clone 2: 2.5 and clone 7: 3.5. The positions of Csk, p52\(^{chk}\), and p56\(^{chk}\) are shown on the left, and those of Csk-Chk and prestained molecular mass markers in kilodaltons are indicated on the right. Exposures: top panel, 21 h; bottom panel, 4 h.
assessed. GAP and its associated proteins (p62 and p190) as well as the adaptor molecule Shc were immunoprecipitated with the appropriate antibodies and probed by anti-phosphotyrosine immunoblotting (Fig. 4, C and D, top panel). Contrary to MEFs expressing p50^csk (lanes 1, 3, and 4), cells lacking Csk (lane 2) exhibited pronounced tyrosine phosphorylation of GAP-associated p62 and p190 (Fig. 4C), as well as of the 52- and 46-kDa isoforms of Shc (Fig. 4D). Little tyrosine phosphorylation of the 120-kDa GAP polypeptide was observed in these cells. Introduction of either Chk isoform in Csk-deficient MEFs dramatically reduced tyrosine phosphorylation of GAP-associated p62 and p190 (Fig. 4C, lanes 5–8). Similarly, the phosphotyrosine content of Shc (Fig. 4D, lanes 5–8) was diminished. Although the tyrosine phosphorylation of these substrates was clearly regulated by Chk, several polypeptides remained slightly hyperphosphorylated, especially in cells expressing p56^chk. This may relate to the lower levels of p56^chk expression generally achieved in these cells (Fig. 2).

Regulation of Src Family Kinases by Chk in Vivo—To further prove that Chk has Csk-like activity in fibroblasts, its influence on the enzymatic activity of Src and Fyn was determined. The cell lines described above were lysed in RIPA buffer, and the enzymatic activity of Src or Fyn was determined in immune complex kinase reactions, using acid-denatured rabbit muscle enolase as a model substrate (Fig. 5, A and C). The relative specific activity of these enzymes was then calculated as described in the legend of Fig. 5. All experiments were conducted under linear assay conditions (data not shown). In agreement with other reports (4, 5, 35), the ability of p60^src (Fig. 5, A and B) and p59^fyn (Fig. 5, C and D) to phosphorylate enolase was increased ~5-fold in MEFs lacking Csk (lane 2), in contrast to MEFs containing p50^csk (lanes 1, 3, and 4). Expression of either p52^chk (lanes 5 and 6) or p56^chk (lanes 7 and 8) in Csk-deficient

**FIG. 3.** Effects of Csk-related enzymes on the abundance of phosphotyrosine-containing proteins in Csk-deficient mouse embryo fibroblasts. The abundance of phosphotyrosine (P.tyr)-containing proteins in the cell lines depicted in Fig. 2A was determined by anti-phosphotyrosine immunoblotting of total cell lysates. The migrations of the major tyrosine-phosphorylated substrates are indicated on the left, whereas those of prestained molecular mass markers in kilodaltons are shown on the right. Exposure, 12 h.

**FIG. 4.** Tyrosine phosphorylation of individual substrates in mouse embryo fibroblast derivatives. Individual tyrosine phosphorylation substrates were recovered from the cell lines depicted in Fig. 2A by immunoprecipitation with the indicated antibodies. Their phosphotyrosine content was then determined by immunoblotting with anti-phosphotyrosine antibodies (top panels). The relative abundance of these proteins was also measured by immunoblotting of parallel immunoprecipitates (IP) with the appropriate antisera (bottom panels). The positions of the various substrates are indicated on the left, whereas those of prestained molecular mass markers in kilodaltons are shown on the right. A, anti-cortactin immunoprecipitates. Exposures: top panel, 2 days; bottom panel, 3 h. B, anti-paxillin immunoprecipitates. Exposures: top panel, 12 h; bottom panel, 2 h. C, anti-GAP immunoprecipitates. Exposures: top panel, 18 h; bottom panel, 3 h. D, anti-Shc immunoprecipitates. Exposures: top panel, 27 h; bottom panel, 4 h.
MEFs reduced the specific activity of p60c-src by 2.5–3-fold, whereas that of p59fyn was diminished by 3–5-fold (Fig. 5, B and D). Once again, this diminution was slightly less than that effected by Csk expression (lanes 3 and 4). We also noted that the abundance of Fyn was increased in cells expressing Csk or Chk (Fig. 5C, lanes 3–8), in comparison with csk−/− MEFs (lane 2). Perhaps, the abundance of p59fyn was lowered in cells lacking Csk, in order to compensate for the augmented specific activity of the enzyme.

**Expression of the Csk-related Enzyme Chk in BI-141 T-cells**—We also tested the impact of Chk on the physiology of BI-141 T-cells. Cells were infected with retroviruses encoding either p52chk or p56chk, in combination with the neomycin resistance gene (neo). Furthermore, a chimera encompassing the full sequence of p52chk and the 15 amino-terminal residues of Src (Src-Chk chimer; Fig. 1) was engineered and also introduced in BI-141 cells. After selection in G418-containing medium, Chk-expressing monoclonal cell lines were detected by immunoblotting with a PhosphorImager and correcting for the abundance of Src (bottom panels)

The relative specific activity of c-Src and Fyn from Csk-deficient MEFs was considered as 1.0.
Src-Chk. p56\(^{chk}\) was also unable to repress IL-2 secretion, except at lower concentrations of anti-TCR antibodies (37 ng/ml). The lack of significant functional impact of the Chk proteins in BI-141 cells was contrary to the effect of Csk and Src-Csk, which dramatically inhibited IL-2 production at all antibody concentrations used (Fig. 7A; Refs. 7 and 8). We also evaluated the effects of Chk on the ability of BI-141 cells to produce IL-2 in response to antigen (bovine insulin) and class II MHC-bearing spleen cells (Fig. 7B). This stimulus is a more physiological means of activating BI-141 cells. While Src-Csk completely blocked antigen/MHC-triggered lymphokine production, none of the Chk proteins, including p56\(^{chk}\), reduced this response.

**Inadequate Recruitment of Chk to Cellular Membranes in BI-141 T-cells**—In order to understand the basis for the inefficiency of Src-Chk at repressing antigen receptor signaling, we examined whether addition of the Src myristoylation signal properly recruited Chk to cellular membranes. BI-141 derivatives expressing Src-Chk or Src-Csk were homogenized in hypotonic buffer, and particulate (P100) and cytosolic (S100) fractions were separated by differential centrifugation. While P100 contained cellular membranes, S100 possessed the cytosolic content. Cell lysates were probed by immunoblotting with anti-Src MAb LA-074 (Fig. 8A). In this assay, lysates corresponding to 4 × lower cell numbers were used for the S100 fraction, to avoid overloading of the protein gel. This factor was taken into consideration in calculating the proportion of proteins present in P100 and S100 (Fig. 8A, right-hand panel). As reported elsewhere (8), Src-Csk was mostly (approximately 90%) positioned in the particulate fraction of BI-141 cells (lane 3). In contrast, however, Src-Chk was primarily (80%) located in the cytosolic fraction (lane 6). Similar results were obtained with other Src-Chk-expressing BI-141 clones (data not shown).

Finally, we examined whether the lack of membrane targeting of Src-Chk was related to a defect in myristoylation. Cells were metabolically labeled with either \(^{3}H\)myristic acid or \(^{35}S\)methionine, and the Src-tagged polypeptides were immunoprecipitated using MAb LA-074. Incorporation of radioactivity was monitored by fluorography (Fig. 8B). Similar to Src-Csk (lanes 2 and 3), Src-Chk (lanes 4 and 5) was efficiently labeled with both \(^{3}H\)myristic acid (top panel) and \(^{35}S\)methionine (bottom panel). In contrast, a variant of Src-Csk in which the site of Src myristoylation (glycine 2) was mutated to alanine
(A2Src-Csk; lane 6) did not incorporate the radioactive lipid (top panel), although it was labeled with [35S]methionine (bottom panel). Together, these results demonstrated that Src-Chk failed to stably associate with cellular membranes even though it was myristoylated. This defect in membrane binding was not caused by mutations in the Src amino terminus, as revealed by careful re-sequencing of the src-chk cDNA (data not shown).

**DISCUSSION**

We and others (11, 12, 18) have shown that the two isoforms of the Chk tyrosine protein kinase (p52<sup>chk</sup> and p56<sup>chk</sup>) can phosphorylate the inhibitory carboxyl-terminal tyrosine of Src family kinases (Lck, Src, and Fyn) in immune complex kinase reactions and in yeast co-expression systems. Even though these phosphorylation assays are notoriously crude, the efficiency of Chk at regulating Src family kinases was roughly equivalent to that of p50<sup>csk</sup>. This finding raised the possibility that, like p50<sup>csh</sup>, the Chk proteins may function in vivo by inhibiting Src family kinases. To begin understanding the function(s) of Chk in mammalian cells, we have evaluated its ability to carry out Csk-like functions in vitro, using two cellular systems in which Csk expression is known to have profound biochemical and/or biological consequences.

Our results demonstrated that p52<sup>chk</sup> and p56<sup>chk</sup> were efficient at repressing phosphotyrosine levels in Csk-deficient MEFs. Seemingly, the Chk kinases could down-regulate all the tyrosine phosphorylation events provoked by the absence of Csk. These involved components of the cytoskeleton such as cortactin, paxillin, Fak, and AFAP-110, as well as molecules implicated in the Ras pathway like Shc and the GAP-associated proteins. The effect of Chk was most likely due to repression of Src family kinases, as the activity of p60<sup>c-src</sup> and p59<sup>fyn</sup> in Csk-deficient MEFs was also diminished by Chk expression. As reported by others (5), however, we were not able to directly study the phosphorylation sites of Src family kinases in these cells, as they contain very low quantities of these enzymes. Nonetheless, on the basis of the documented activity of Chk against Src family kinases in vitro (11, 12, 18), it is fair to assume that Chk mediated these effects in csk<sup>−/−</sup> MEFs by...
inactivating Src family kinases. Hence, in combination, these data provided firm evidence that Chk exhibits Csk-like activity not only in vitro but also in vivo. The impact of Chk in MEFs was consistently less marked than that of p50
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. Since the abundance of Chk and Csk in these cells was roughly comparable, this raised the possibility that the intrinsic enzymatic activity of Chk may be lower than that of Csk, even though this notion may not be supported by earlier studies of their catalytic activity in vitro. Alternatively, it is plausible that Chk is recruited less efficiently in the vicinity of activated Src family kinases, as a consequence of differences in the affinity and/or specificity of its SH3 or SH2 domains. Or, Chk may not be able to physically interact with other proteins that normally participate in the function of Csk in cells. In keeping with this idea, we recently demonstrated that the SH3 regions of Csk and Chk have dramatically distinct binding specificities in vitro and in vivo (26). Finally, Chk may be physically restricted to a specific cellular compartment, thereby being unable to regulate all pools of Src family kinases in Csk-deficient MEFs. Further support for this concept will be presented below.

The capacity of Chk to behave as a negative regulator of antigen receptor signaling in T-cells was also evaluated. Even though the Chk proteins could be expressed in amounts comparable with those of p50
\textsuperscript{chkb}
, the Chk proteins could be expressed in amounts comparable with those of p50
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, this raised the possibility of a singular ability of Chk to accumulate in certain cellular compartments or to a yet unappreciated capacity to phosphorylate cellular targets other than Src family kinases.

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