Growth Factors

ISSN: 0897-7194 (Print) 1029-2292 (Online) Journal homepage: http://www.tandfonline.com/loi/igrf20

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To cite this article: Svetozar Grgurevich, Diana Linnekin, Tiziana Musso, Xiaoying Zhang, William Modi, Luigi Varesio, Francis W. Ruscetti, John R. Ortaldo & Daniel W. McVicar (1997) The Csk-like proteins Lsk, Hyl, and Matk represent the same Csk homologous kinase (Chk) and are regulated by stem cell factor in the megakaryoblastic cell line MO7e, Growth Factors, 14:2-3, 103-115, DOI: 10.3109/08977199709021514

To link to this article: http://dx.doi.org/10.3109/08977199709021514

Published online: 11 Jul 2009.

Growth Factors, 1997, Vol. 14, pp. 103–115
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The Csk-like proteins Lsk, Hyl, and Matk represent the same *C*sk *h*omologous *k*inase (Chk) and are regulated by stem cell factor in the megakaryoblastic cell line MO7e

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(Received on 9 August 1996, in final form 30 August 1996)

Recently, the cDNAs for Lsk, Matk and Hyl, three Csk-related protein tyrosine kinases, have been cloned. We have examined the relationship of Lsk, Matk and Hyl, and found that the gene for each of these proteins is localized to the same region of human chromosome 19. Further, the proteins encoded by Lsk and Matk cDNAs are immunologically similar. These data strongly suggest that Lsk, Hyl and Matk are the same gene product. Previous reports demonstrating expression of Hyl and Matk in hematopoietic lineages led us to investigate the regulation of Lsk expression in response to stem cell factor (SCF) and granulocyte-macrophage colony stimulating factor (GM-CSF) in M07e, a human leukemic cell line. Induction of Lsk/Hyl/Matk protein and mRNA was observed after treatment with SCF but not with GM-CSF. GM-CSF and IL-3, potent mitogens, had no effect on Lsk/Hyl/Matk expression. In contrast, PMA induced Lsk/Hyl/Matk but did not stimulate proliferation. Therefore, induction of Lsk/Hyl/Matk does not correlate with the capacity to stimulate proliferation. None of the stimuli examined increased Csk protein or mRNA expression. These data demonstrate differential regulation of Csk family members by cytokines and suggest a role for Lsk/Hyl/Matk in responses mediated by SCF and PMA. Further, our data demonstrate that, as has been seen in blood monocytes, cytokine driven translational control of Lsk/Hyl/Matk is likely a critical mode of regulation. Lastly, since our studies strongly suggest that the Lsk, Hyl and Matk kinases are related and regulated distinctly from Csk, we and several of the original authors have agreed to rename this kinase the *C*sk *h*omologous *k*inase (Chk).

Keywords: Kinase, stem cell growth factor, src kinase

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INTRODUCTION

The role of protein tyrosine phosphorylation in leukocyte growth, signal transduction, and differentiation is well established. One of the most prominent families of non-receptor protein tyrosine kinases (PTK) is the src family. This family consists of 9 highly related members that exhibit patterns of expression ranging from the broadly expressed Yes and Src, to the more tissue restricted expression pattern of Lyn and Blk (Reviewed by Mustelin, 1994).

Regulation of src-family members has been studied extensively, particularly in relation to enzymatic activity (Cooper and Howell, 1993). Csk, a 50 kDa cytoplasmic PTK, mediates phosphorylation of a highly conserved tyrosine residue within the carboxyl-terminus of src-family members (tyrosine 527 in c-src) resulting in a dramatic reduction in enzymatic activity (Okada *et al.*, 1991; Nada *et al.*, 1991). The importance of this regulatory mechanism is underscored by the existence of an oncogenic form of src that lacks the Csk phosphorylation site (Jove and Hanafusa, 1987). Recently, we have cloned a protein tyrosine kinase related to Csk, that has a highly restricted pattern of expression (McVicar *et al.*, 1994). This kinase, termed Lsk (leukocyte C-terminal src kinase), is 52.8% identical to Csk, and, like Csk, contains SH2 and SH3 domains. Our initial characterization of Lsk demonstrated expression in brain, natural killer cells and activated T cells (McVicar *et al.*, 1994). Studies from our laboratory have also shown expression of Lsk in IL-4 or IL-13 stimulated peripheral blood monocytes, a phenomenon blocked by coculture of monocytes with IFN-γ (Musso *et al.*, 1994).

In addition to Lsk, two other human Csk-like cDNA sequences have been reported. However compared to Lsk, these cDNAs, termed Matk (megakaryocyte associated tyrosine kinase) (Bennet, 1994) and Hyl (hematopoietic consensus tyrosine lacking kinase) (Sakano, 1994), have different reported patterns of expression and predicted polypeptides. Despite these differences, the high cDNA sequence homology to Lsk raises the intriguing possibility that Lsk, Matk and Hyl may all represent the same gene. If true, the documented cytokine regulation of Lsk in monocytes (Musso *et al.*, 1994) together with the description of Hyl and Matk expression in hematopoietic cells (Bennet, 1994; Sakano, 1994) would suggest potential regulation of this new Csk family member by hematopoietic growth factors.

Here we have examined the relationship of Lsk to the Matk gene product, and found that the proteins encoded by Lsk and Matk cDNAs are immunologically related. Further, we have found that the genes for Lsk, Matk and Hyl are all localized to the same region of human chromosome 19. Together these data strongly suggest that Lsk, Matk and Hyl represent the same gene product. We have also examined regulation of Lsk/Hyl/Matk in response to stem cell factor (SCF) and granulocyte/macrophage-colony stimulating factor (GM-CSF) in the megakaryolastic cell line M07e. SCF (also termed mast cell growth factor, steel factor and c-kit ligand) promotes survival, proliferation and differentiation in hematopoietic progenitor cells and is also a potent mast cell growth factor (Witte, 1990). Similarly, GM-CSF stimulates proliferation and differentiation of progenitor cells as well as activation of neutrophils and monocytes (Reviewed by Gasson, 1991). M07e cells proliferate in response toboth GM-CSF and SCF, are CD34 positive and serve as a good model for the study of signal transduction pathways of hematopoietic growth factors in progenitor cells (Avanzi *et al.* 1990; Hendrie *et al.*, 1991). Our studies demonstrated that SCF, but not GM-CSF, treatment of M07e cells induced expression of Lsk/Hyl/Matk mRNA and protein. In contrast, Csk protein and mRNA levels were largely unaffected by these growth factors. Interestingly, Lsk/Hyl/Matk expression did not correlate with cell proliferation. PMA strongly induced Lsk/Hyl/Matk expression in the absence of a significant proliferative response. These data suggest that Lsk/Hyl/Matk plays a role in the cellular responses mediated by SCF and PMA, and demonstrate that the two known members of the Csk family of tyrosine kinases are differentially regulated in reponse to diverse stimuli.

MATERIALS AND METHODS

Cells, cytokines and antibodies

M07e cells were maintained in RPMI 1640, 10% fetal calf sera, 2 mM L-glutamine and 1% penicillin-streptomycin (cell culture media) supplemented with recombinant human GM-CSF (10 ng/ml) and recombinant human stem cell factor (100 ng/ml). Jurkat cells were maintained in RPMI-1640 supplemented with 2 mM glutamine, 10% fetal calf serum, and antibiotics. After informed consent, natural killer cells (NK) were prepared from the peripheral blood of healthy volunteers as described previously (Ortaldo *et al.*, 1986). Human GM-CSF, SCF, IL-4 and IL-3 were purchased from PeproTech (Rocky Hill, NJ). Human recombinant IFN-γ (lot NN9027AX, specific activity 2.02×10^7 U/ mg) was kindly provided by Dr. H.M. Shephard (Genentech Labs, San Francisco, CA). Recombinant purified IL-2 from Escherichia coli, (lot LP-381, sp. act. of 18×10^6 IU/mg, and LPS content of less than 0.0006 ng/ml) was kindly provided by Cetus Corporation (Emeryville, CA). Antibodies to Lsk included both a polyclonal rabbit serum (McVicar *et al.*, 1994; Musso *et al.*, 1994) and a monoclonal antibody (4F6A5) generated against the predicted carboxyl- terminal of the Lsk protein (AA 449–465). Csk antibody was kindly provided by Dr. Marietta Harrison (Purdue University) and the N-terminal Matk antibody, Sald03, was provided by Dr. Hava Avraham of Harvard Medical School.

Northern blot analysis

Cells were lysed in guanidinium isothiocyanate and total RNA was isolated by sedimentation through a CsCl gradient (Chirwing *et al.*, 1979). Northern Blot analysis was performed as previously described (Musso *et al.*, 1992). In brief, 20 μg of total RNA was electrophoresed in a 0.8% agarose-formaldehyde gel and transferred by capillary action onto Nytran (Schleicher and Schuell, Keen, NH). In all experiments equal loading of the RNA was confirmed by ethidium bromide staining. The cDNA probe used was the KpnI/ KpnI fragment of the Lsk cDNA or the complete cDNA of the chicken beta actin gene. After hybridization blots were washed twice at high stringency (0.2 m × SSC, 0.1% SDS, 65°) and exposed to film at –70°C with intensifying screens.

Immunoprecipitations, electrophoresis and Western blot analysis

M07e cells were lysed in buffer containing 0.5% Triton-X 100, 300 mM NaCl, 50 mM Tris (pH 7.4) 2 mM EDTA, 1 mM Na_3VO_4, 10 mM sodium fluoride, 1μg/ml leupeptin, 1μg/ml aprotinin, and 2.5 μM p-nitrophenyl-p-guanidino-benzoate. Post-nuclear supernatants were assayed for protein content by the BCA method as directed by the manufacturer (Pierce) using BSA as a standard. In all cases aliquots of cell lysates containing equal amounts of protein were boiled in Laemmli sample buffer and subjected to electrophoresis through 8 or 10% polyacrylamide gels and electrophoretically transferred to Immobilon. The resulting blots were probed with the indicated antibody as previously described. Bound antisera was detected using horseradish peroxidase conjugated goat anti-rabbit antisera (Boehringer Mannheim) followed by detection with enhanced chemiluminescence (ECL, Amersham). Immunoprecipitations of cell lysates were performed for 1 h at 4°C with monoclonal 4F6A5 that had been precoupled to protein G sepharose. Immunoprecipitates were washed 4 times with wash buffer containing 0.1% triton X100, and bound proteins were eluted with Laemmli sample buffer.

Reverse transcription and PCR amplification

Total RNA was isolated from peripheral NK cells using RNAzol (Cina/Biotecx, Inc., Frendswood, TX). 1μg of RNA was reverse transcribed using a combination of 2 Lsk primers (5'-GGC CAG CTC CCC TGG CTT-3' and 5'-TCC TCG GGA GGC TGC AGC-3') with AMV reverse transcriptase (Gibco BRL,) for 1 h at 37°C. A control reaction included reverse transcriptase but was heat inactivated at 65°C for 10 min prior to incubation at 37°C. The resulting reactions (25 μl) were cleared of primers by washing through a Centricon 10 column in a total of 3 ml water. The eluted cDNA (150μl) was stored at 4°C prior to use in PCR reactions.

PCR amplification was carried out using 3.5μl of cDNA template in a 100μl reaction containing an Lsk derived down stream primer (5'-GTG TGC TCG CAT TTG GTG ATA C-3') and an Matk derived upstream primer (5'-ATG CGG ATC C TG TGG CAG GCC ATT CCC AGC-3') containing a BamHI linker (underlined). A control reaction contained no cDNA template. Reactions were amplified for 3 cycles as follows, 1 min at 96°C, 1 min at 50°C, 1 min at 72°C, then 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. Final extension was at 72°C for 10 min. The resulting products were electrophoresed in 0.8% agarose and visualized by ethidium bromide staining. No product was obtained with the heat inactivated reverse transcriptase or from the PCR reaction with no template. The product was subcloned using the TA cloning kit (Invitrogen) and sequenced using the Sequenase based dideoxy chain termination method (USB). All sequence data was analyzed using the programs of the Genetics Computer Group of the University of Wisconsin.

Transfections

The missing 5'AUG was engineered on to the Lsk cDNA using PCR. The forward primer was 5'- ATG GGA TTC ATG GCG GGG CGA-3' and the reverse primer was 5'-AAG GAT CCA CTC TCT CGG TCC TCT GG-3'. Each primer contained a BamHI linker (underlined). The resulting product was digested with BamHI and subcloned into the pGEM-11Zf vector. The KpnI/KpnI fragment of the PCR product was then removed from pGEM-11Zf and replaced with the KpnI/KpnI fragment from the original cDNA to eliminate possible PCR generated errors. The full construct was then removed by BamHI digestion and cloned into the BamHI site of the beta actin mammalian expression vector LK588. The Matk cDNA (kindly provided by Dr. Hava Avraham, Harvard Medical School) (Bennet *et al.*, 1994) was removed by EcoRI digestion and subcloned into the EcoRI site of the CMV driven expression vector pcDNA3 (Invitrogen). The resulting constructs or empty LK588 vector were transfected into COS-7 cells using DEAE-Dextran. After 48 h the cells were harvested, lysed and blotted with anti-Lsk antibody (AB266) as described above.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was performed as described previously (Tory *et al.* 1992). The Lsk gene was represented by cDNA spanning the complete open reading frame of Lsk. These probes were used as probes for *in situ* hybridization using standard fluorescent technique. In brief, metaphase chromosomes were prepared from PHA-stimulated peripheral blood leukocytes. Probe DNA was labeled with biotin-11-dUTP using nick translation. Hybridization (50 ng/ml probe final) was performed at 37°C for 16 h followed by washes at 40°C in a solution containing 50% formamide in 2×SSC. Slides were incubated in a detection solution containing 5μg/ml fluorescein isothiocyanate (FITC)-conjugated avidin. Photographs were taken with Kodak EKTAR 1000 colorfilm.

Proliferation assays

M07e cells were washed two times and resuspended at 10^5/ml in cell culture media. Cells were aliquoted into 96 well plates and incubated 72 h (37°C, 5% CO_2) in the presence of either media or the indicated growth factors. Each well was pulsed with 1μCi of ^{3}H-thymidine (6.7 Ci/mM, NEN, Boston, MA) for 6–8 hours and then harvested (Scatron Semiautomatic Cell Harvester) onto glass fiber filter paper (Filtermat, Skatron, Inc., Sterling, VA). Filter strips were dried and counted in a liquid scintillation counter (Model 1216, LKB, Piscataway, NJ).

RESULTS

Lsk, Matk and Hyl represent the same gene product

The cDNA sequences reported for Lsk, Matk and Hyl are very similar yet the reported predicted polypeptides differ significantly (Bennet *et al.*, 1994; McVicar *et al.*, 1994; Sakano *et al.*, 1994). The cDNAs for both Matk and Hyl encode 41 amino acids upstream of the predicted start site of Lsk and the predicted carboxyl-terminus of Matk differs markedly from that of Lsk

and Hyl. In addition, the murine homolog to these genes (Ctk/Ntk) has been reported to exist in two isoforms; one isoform contains the residues upstream of the reported Lsk start site while the other does not (Chow *et al.*, 1994a; Chow *et al.*, 1994b; Klages *et al.*, 1994). In order to define the structural relationship of human Lsk and Matk, we used a monoclonal antibody specific for a peptide sequence in the carboxy-terminus of the predicted Lsk protein. Lsk was immunoprecipitated with this antibody and immunoblotted with the Lsk-specific polyclonal antiserum we have previously described (McVicar *et al.*, 1994; Musso *et al.*, 1994). The left panel of Fig. 1A demonstrates that the monoclonal antibody recognizing the predicted carboxy-terminus of Lsk specifically immunoprecipitated the Lsk protein. The same monoclonal antibody was next used to examine the relationship of Lsk to Matk. Lsk was immunoprecipitated with the carboxy-terminal monoclonal antibody and subsequently immunoblotted with Sald03, an antiserum specific for the amino terminus of Matk (Fig. 1A, right panel) (Bennet *et al.*, 1994). The Matk antibody specifically recognized Lsk in NK cells and M07e cells but not in the Lsk negative Jurkat T cell line. Together these data suggest that the polypeptide sequence of Lsk contains the carboxy-terminus reported for Lsk and the N-terminus reported for Matk. The resulting hybrid polypeptide would match the predicted amino acid sequence of Hyl, a cDNA for which protein expression has yet to be described (Sakano *et al.*, 1994).

If the Lsk gene encodes the N-terminal residues reported for Matk, then NK cells, an abundant source of Lsk, should contain mRNA corresponding to the N-terminal and 5' untranslated region of Matk. To directly address this possibility we isolated total RNA from highly purified natural killer cells, produced Lsk cDNA by reverse transcription (RT) with Lsk specific primers and then performed nested PCR using a downstream primer from Lsk and an upstream primer derived from Matk. The result was a 250 bp RT-dependent product which when subcloned and sequenced was found to be identical to the reported 5' region of Matk (data not shown) (Bennet *et al.*, 1994). These studies demonstrate that Matk/Hyl 5' mRNA is expressed in NK cells.

The gene for Hyl has previously been mapped to human chromosome 19p13.3 (Sakano *et al.*, 1994). Similarly, Matk has been localized to chromosome 19 (Avraham, 1995). We have now mapped the chromosome localization of Lsk using fluorescence *in situ* hybridization (FISH) (Tory *et al.*, 1992). cDNA probes from Lsk were labeled using biotinylated dUTP, and detected using fluorescein isothiocyanate-conjugated avidin. Chromosome identification was carried out using QFH-banding by simultaneous Hoechst 33258 staining (data not shown). Following hybridization, a total of 94 metaphase cells were examined. Thirty of these cells exhibited paired hybridization signals and an additional 24 cells showed one hybridization signal at 19p13.3 (Fig. 1B). These data suggest that Lsk, Hyl, and Matk localize to the same region of human chromosome 19.

To further address the relationship of the Matk and Lsk primary peptide structure, the cDNAs were cloned into expression vectors and transfected into COS-7 cells. After 48th the cells were harvested, lysed and immunoblotted with anti- Lsk antiserum (Fig. 1C). Anti-Lsk antiserum detected both the Matk and Lsk proteins confirming that the Matk cDNA encodes a protein immunologically identical to Lsk.

Differential regulation of Lsk/Hyl/Matk and Csk in response to SCF and GM-CSF

Lsk/Matk/Hyk has been found in megakaryoblastic cell lines as well as CD34 positive progenitor cells and we have previously demonstrated its regulation by cytokines in monocytes (Bennet *et al.*, 1994; Sakano *et al.*, 1994; McVicar *et al.*, 1994; Musso *et al.*, 1994). Therefore, we examined the regulation of Lsk/Hyl/Matk in the megakaryoblastic cell line M07e. Comparable to hematopoietic progenitor cells, M07e proliferate in response to either IL-3, SCF or GM-CSF alone. In addition, simultaneous stimulation with both GM-CSF and SCF often results in synergistic proliferative responses of either progenitors or M07e

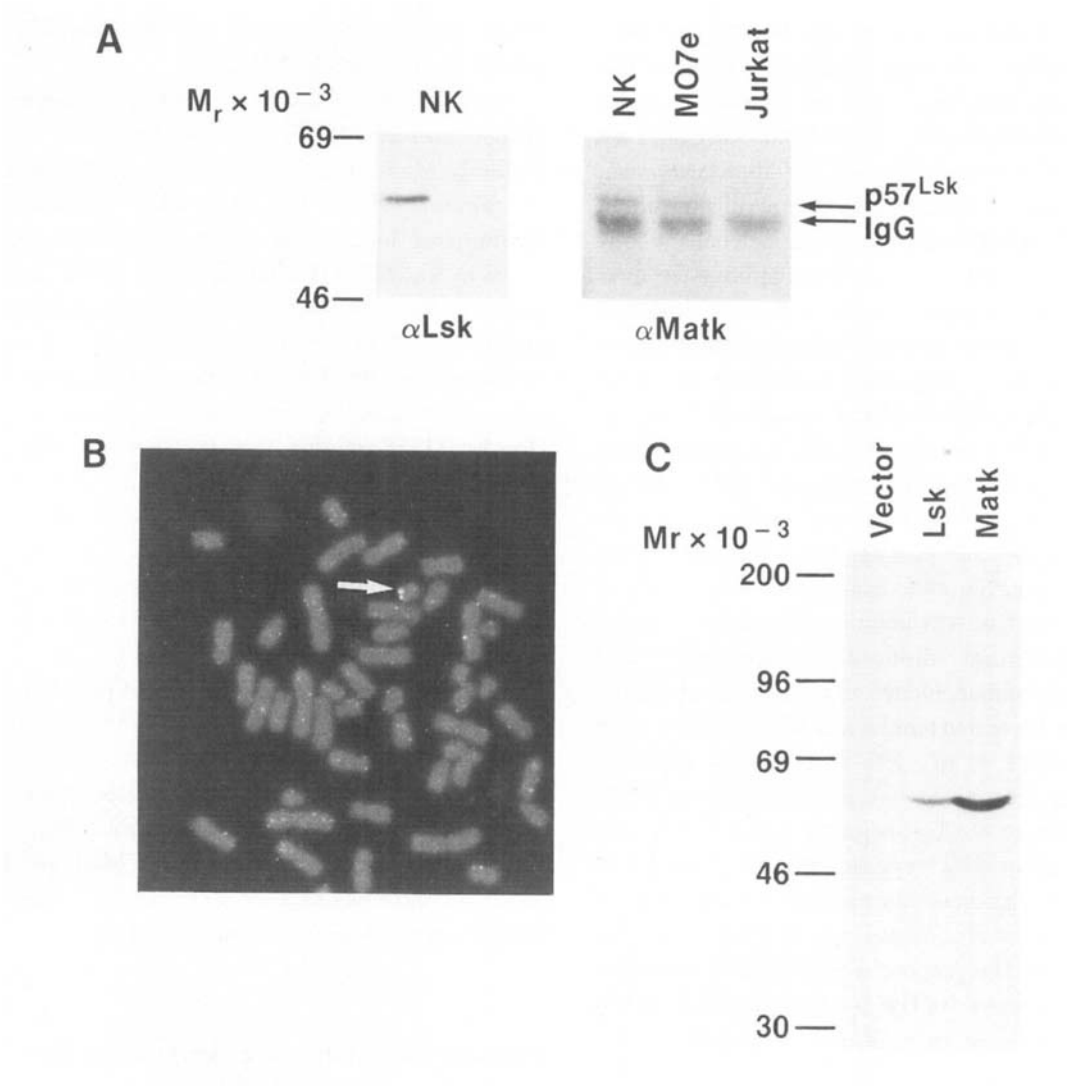

FIGURE 1 A. Immunoprecipitated Lsk is recognized by the Matk antibody Sald03. Left panel: clarified lysate from 107 natural killer cells was immunoprecipitated with the anti-Lsk monoclonal antibody 4F6A5 (lane 1) or a control Mab (lane 2) precoupled to protein G sepharose. The resulting proteins were immunoblotted with anti-Lsk C-terminal polyclonal antibody AB667. Right panel: Lsk was immunoprecipitated as above from Lsk^+ natural killer cells or M07e, and from Lsk^- Jurkat cells. The resulting protein was immunoblotted with anti-Matk N-termal antibody (Sald03). B. A representative partial human metaphase spread showing the specific site of hybridization to chromosome 19. Hybridization with the Lsk cDNA probes visualized with FITC-avidin. The arrow indicates hybridization at 19.p13.3. C. Expression of Lsk and Matk in COS-7. Empty expression vector (lane 1), Lsk expression vector (lane 2) or Matk expression vector (lane 3) was transfected into COS-7 cells with DEAE-Dextran. After 48 h the cells were lysed and subjected to Western blot analysis using an anti-Lsk polyclonal antiserum.

(Avanzi *et al.*, 1990; Hendrie *et al.*, 1991). To determine the effect of SCF and GM-CSF on Lsk/Hyl/Matk expression, M07e cells were incubated for 18 h in media or media supplemented with SCF, GM-CSF or both SCF and GM-CSF. Cells were harvested, lysed and equivalent amounts of protein immunoblotted with anti-Lsk sera. As shown in Fig. 2A, SCF induced expression of the 57 kDa Lsk/Hyl/Matk. The minimal GM-CSF induction of Lsk/Hyl/Matk was not a consistent finding. Interestingly, the combination of

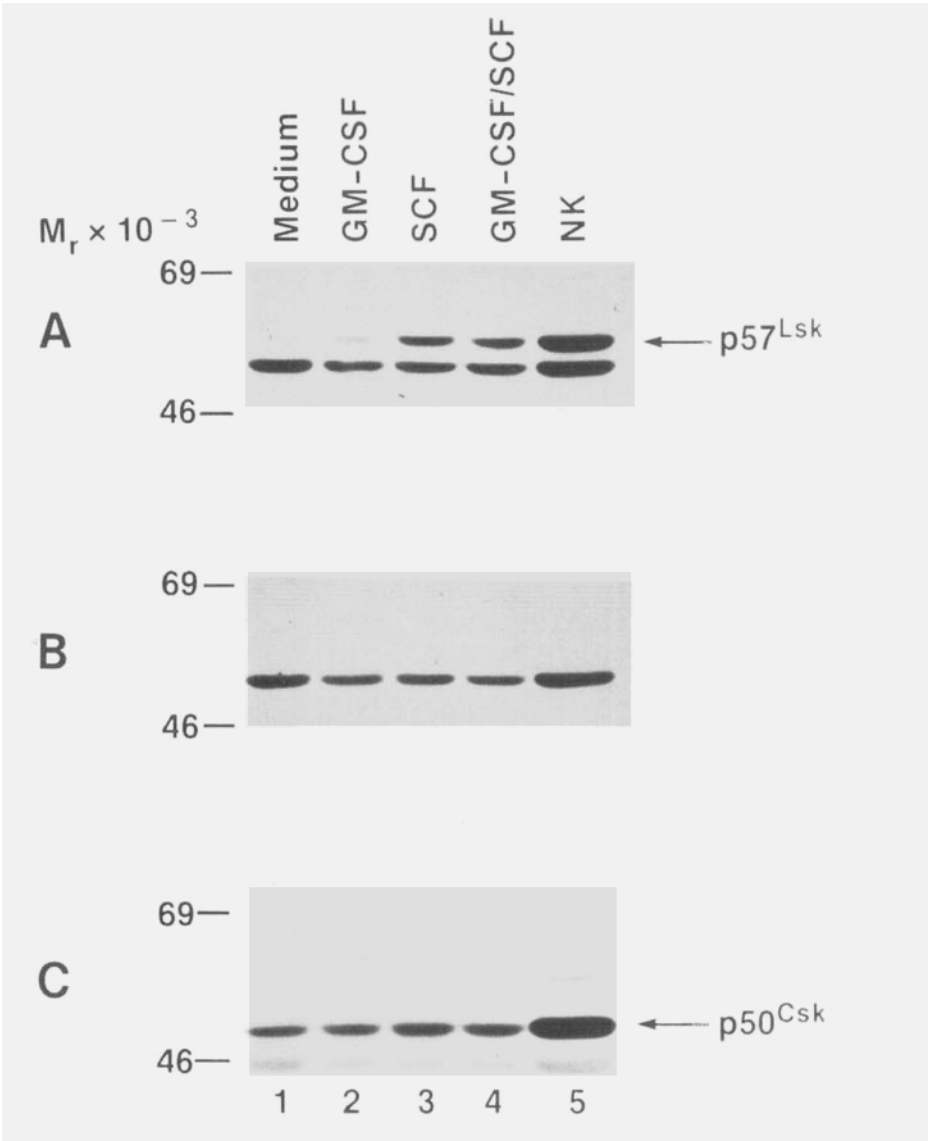

FIGURE 2 Differential Regulation of Csk and Lsk in Response to SCF abnd GM-CSF. M07e cells were deprived of growth factors overnight, washed, resuspended in RPMI 1640 media with 10% fcs, and incubated in the presence or absence of SCF (100 ng/ml), GM-CSF (10 ng/ml) or SCF and GM-CSF. Eighteen hours after stimulation, the cells were harvested, washed and lysed. Equivalent amounts of protein (100 μg) were loaded on a SDS gel, resolved and transferred to Immobilon. Lysates from NK cells (designated NK in Fig. 3) were run as a positive control for the Lsk protein. Panel A. Western blot analysis for Lsk. Panel B. Peptide competition of $p57^{Lsk}$. Panel C. Western blot analysis of Csk protein.

factors did not synergistically induce Lsk/Hyl/Matk expression. The specificity of the antisera for Lsk/Hyl/Matk was demonstrated by the absence of the 57 kDa band in the presence of excess immunogen (Fig. 2B). We also examined the effect of SCF and GM-CSF on expression of Csk (Fig. 2C). Csk was expressed at a constitutively high level in M07e cells and treatment with SCF did not significantly modulate its expression. Because the response to SCF was significantly greater then GM-CSF, we choose to further characterize

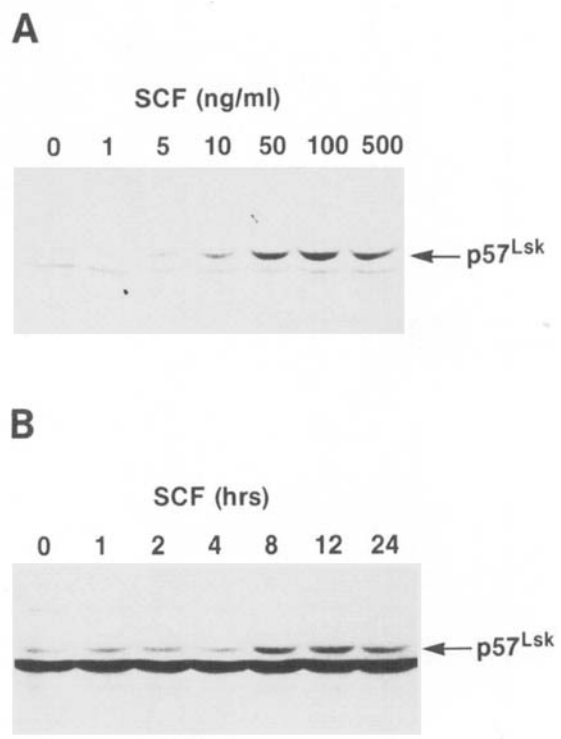

FIGURE 3 SCF-Induced Lsk Expression is Dose and Time Related. Panel A. Dose-response curve for SCF-induced Lsk upregulation. M07e cells were starved overnight, stimulated 18 h with the indicated concentration of SCF and lysed. Equivalent amount of proteins were resolved with SDS-PAGE, transferred to Immobilon and immunoblotted with anti-Lsk sera. Panel B. Cells were starved overnight, stimulated the indicated time with 100 ng/ml SCF, lysed and immunoblotted as described in Panel A.

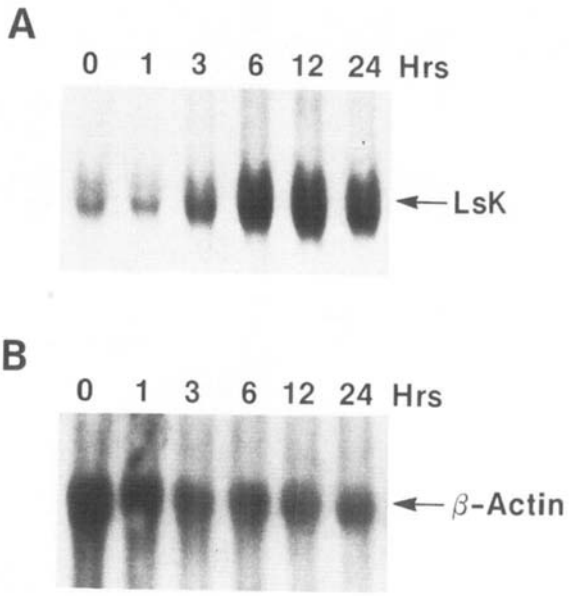

FIGURE 4 Lsk regulation at the mRNA level. A) M07e cells were growth factor starved overnight and then stimulated with 100 ng/ml SCF for the indicated times. Total RNA was extracted and 20ngmg was electrophoresed in a agarose-formaldehyde gel then blotted with an Lsk cDNA probe. B) After stripping, the same filter was analyzed for RNA loading by hybridization with a probe for beta actin.

Lsk/Hyl/Matk induction in SCF treated cells. We found Lsk/Hyl Matk induction to be dose-related with maximum response noted between 50 and 100 ng/ml of SCF (Fig. 3A). This corresponds closely to the dose-response curve for SCF-induced proliferation (data not shown and Hendrie *et al.*, 1991). The time-course for Lsk/Hyl/Matk induction was next examined. Fig. 3B demonstrates that SCF- induced Lsk/Hyl/Matk expression was detectable by 8 h, maximal by 12 h and remained elevated through 24 h. Cultures that continue through 48 h show a reduction in Lsk/Hyl/Matk to near baseline levels (data not shown).

Regulation of Lsk/Hyl/Matk mRNA in response to SCF

To determine if the increases in Lsk/Hyl/Matk protein expression were paralleled by mRNA increases we examined the effect of SCF on Lsk/ Hyl/Matk mRNA expression (Fig. 4A). Stimulation of M07e cells with SCF resulted in Lsk/Hyl/ Matk mRNA accumulation in as little as 3 h. The level of mRNA peaked by 6 h and was maintained throughout the course of the study. Comparable loading of RNA was demonstrated by hybridization of the same filter with a probe for beta actin (Fig. 4B).

Induction of Lsk/Hyl/Matk does not correlate to cytokine-induced proliferation

Our studies have demonstrated that the mitogenic cytokine SCF, induced Lsk/Hyl/Matk expression. We

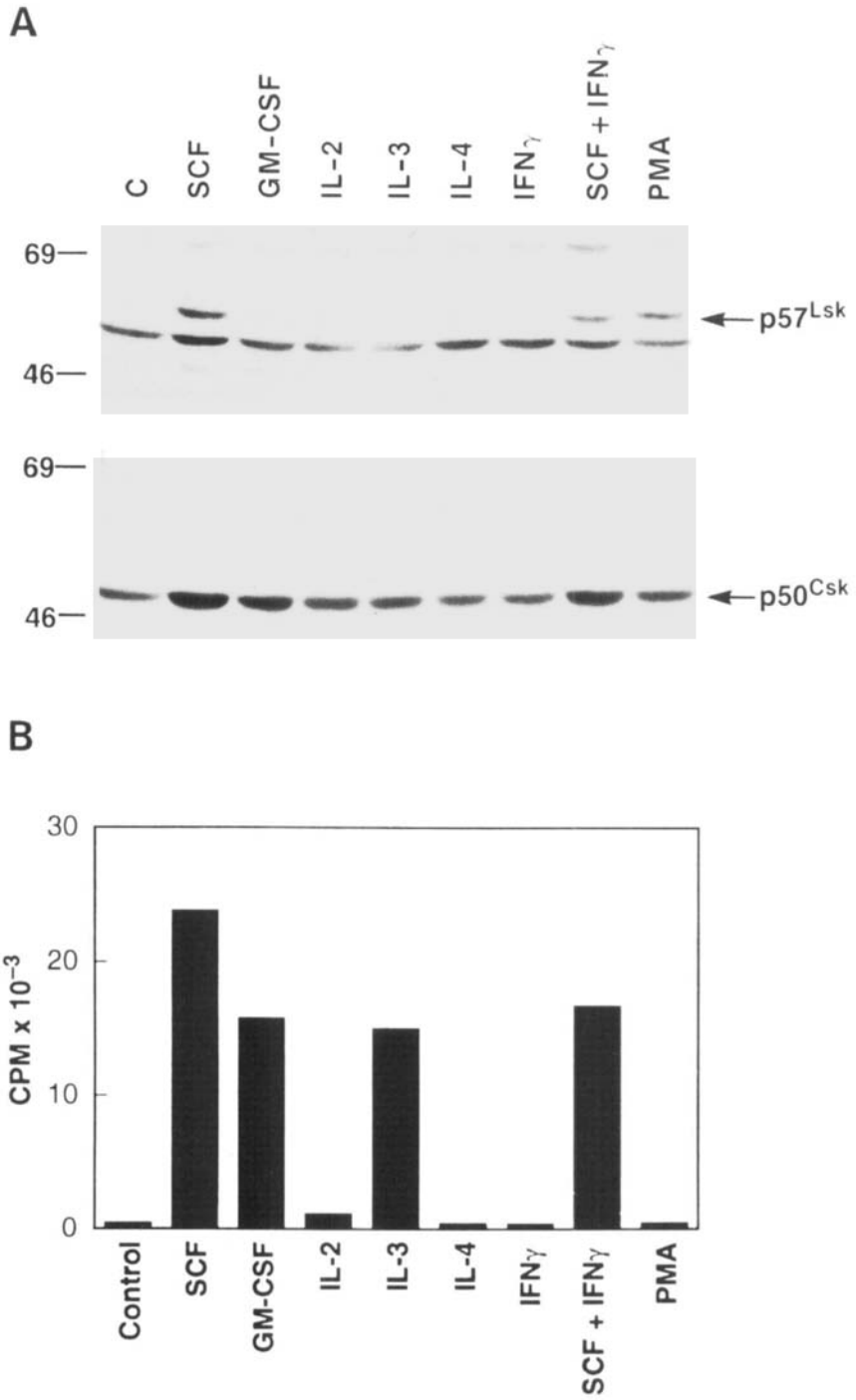

FIGURE 5 Induction of Lsk Does Not Correlate to Cytokine-Induced Proliferation. A) Lsk immunoblot (Upper Panel) and Csk immunoblot (Lower Panel). M07e cells were starved overnight, cultured 18 h with the indicated stimuli, lysed and processed as described in Fig. 3. B) Proliferation results. M07e cells were incubated 72 h with the indicated stimuli in 96 well plates, pulsed 6 h with ^{3}H-thymidine and harvested onto glass filter fiber paper. Data represent the mean of triplicate points.

next addressed whether Lsk/Hyl/Matk induction correlated with cell proliferation. Shown in Fig. 5 are the results of a study comparing the capacity of a variety of stimuli to induce Lsk/Hyl/Matk protein (Fig. 5A upper panel), Csk protein (Fig. 5A lower panel), and proliferation (Fig. 5B) in M07e cells. SCF, GM-CSF, IL-3, and to some extent IL-2, all induced M07e proliferation while IL-4, IFN-γ and PMA did not. In contrast, Lsk/Hyl/Matk was induced by SCF or PMA, but not by GM-CSF, IL-2, IL-3, IL-4 or IFN-γ. Lastly, in contrast to its ability to block IL-4 induced Lsk/Hyl/Matk in monocytes, IFN-γ did not antagonize the ability of SCF to induce Lsk/Hyl/Matk in M07e. These data demonstrate a clear dissociation of induction of Lsk/Hyl/ Matk from proliferative responses of this megakaryoblastic line.

DISCUSSION

Csk is the first member identified of an apparent family of protein tyrosine kinases that phosphorylate the carboxyl-terminus of c-src and src family members (Nada *et al.*, 1991; Okada *et al.*, 1991; Partanen *et al.*, 1991; Bergman *et al.*, 1992; Brauninger *et al.*, 1992). Subsequently, we and others have cloned protein tyrosine kinases that appear related to Csk. Csk-related cDNAs cloned from human libraries include Lsk, Matk and Hyl (Bennet *et al.*, 1994; McVicar *et al.*, 1994; Sakano *et al.*, 1994). In addition, using mouse cDNA libraries, Ntk and Ctk have been identified as potential members of a Csk family (Chow *et al.*, 1994; Klages *et al.*, 1994). Lastly, a Csk-like cDNA from rat brain (Batk) has been reported (Kuo *et al.*, 1994). Although the sequence homology of the human cDNAs is very high, different predicted polypeptide sequences and patterns of expression have been reported. The studies reported here addressed the relationship of the human cDNAs Lsk, Matk and Hyl. Several lines of evidence suggest that Lsk, Matk, and Hyl represent the same gene product. First, the sequence homology of the three cDNAs is greater than 95%. Second, all three genes have now been now been localized to chromosome 19p13.3 (Fig. 1B) (Sakano *et al.*, 1994; Avraham *et al.*, 1995). Third, a monoclonal antibody directed against the carboxyl-terminus of Lsk immunoprecipitates a protein recognized by Matk specific N-terminal antisera. This demonstrated the existence of the Matk/Hyl 5' end in Lsk. Fourth, polyclonal antisera specific for the C-terminal of Lsk recognized both Lsk and Matk when transiently expressed in COS-7. Lastly, reverse transcription of Lsk mRNA from NK cells (the original source of Lsk) demonstrated that the N-terminal sequences of Matk and Lsk are identical. Thus, the protein product of the Lsk/Matk/Hyl, and presumably Batk, genes contain the carboxyl-terminus reported for Lsk and the amino terminus reported for Matk. In total, these data strongly suggest that Lsk, Hyl, Matk, and Batk are the same gene product.

Thus far, Lsk/Matk/Hyl has been reported in brain and activated lymphocytes as well as megakaryocytic and monocytic cells (Bennet *et al.* 1994; McVicar *et al.*, 1994; Sakano *et al.*, 1994; Musso *et al.*, 1994). The presence of this PTK in hematopoietic cells, together with its regulation by cytokines in peripheral monocytes, led us to examine Lsk/Hyl/Matk regulation in response to a number of hematopoietic growth factors. We have found that stimulation of the M07e cells with SCF dramatically increases the expression of both Lsk/Hyl/Matk mRNA and protein. These data are in apparent agreement with the reported interaction between c-Kit and Lsk/Hyl/Matk in lines that constitutively express the kinase (Jhun *et al.*, 1995). GM-CSF failed to consistently induce Lsk/Hyl/Matk expression. Interestingly, treatment of M07e cells with both SCF and GM-CSF often resulted in synergistic proliferative responses (Avanzi *et al.*, 1990; Hendrie *et al.*, 1991), yet we have not observed synergistic induction of Lsk/Hyl/Matk. In addition to SCF, we found PMA to be a potent inducer of Lsk/ Hyl/Matk protein expression. These findings confirm previous reports demonstrating PMA- induced upregulation of Matk and Hyl mRNA in megakaryoblastic lines (Bennet *et al.*, 1994; Sakano *et al.*, 1994). Our findings with PMA, however, are of particular note, since, in contrast to SCF, PMA did not stimulate M07e proliferation. Contrary to other reports (Avraham *et al.*, 1995), our

finding that IL-3 and GM-CSF induce proliferation but not Lsk/Hyl/Matk expression, demonstrates that Lsk/Hyl/Matk induction is not involved in the proliferative response.

In previous studies, we found that stimulation of peripheral monocytes with IL-4 resulted in increased expression of Lsk/Hyl/Matk (Musso *et al.*, 1994). The present study demonstrates that IL-2, IL-4 and IFN-γ did not induce Lsk/Hyl/ Matk in M07e cells. These findings are of note because IL-4 stimulates phosphorylation of the Janus kinases JAK1 and JAK3 in both monocytes and M07e cells (Musso *et al.*, 1995). Thus, activation of the JAK-STAT signal transduction pathway per sei does not appear to be sufficient for Lsk/Hyl/Matk induction. Further complexity in the regulation of Lsk/Hyl/Matk is suggested by the difference in IFN-γ on IL-4 induced upregulation of Lsk/Hyl/Matk in monocytes versus M07e cells. Though IFN-γ potently inhibited IL-4-induced upregulation of Lsk/Hyl/Matk in monocytes, little effect was observed during SCF- induced Lsk/Hyl/Matk regulation in M07e cells (Fig. 5) (Musso *et al.*, 1994). Together these findings point to cell/lineage specific regulatory mechanisms of Lsk/Hyl/Matk as opposed to cytokine or stimulus specific pathways.

Regulation of Lsk/Hyl/Matk expression in hematopoietic cells is intriguing in light of the dramatic regulation of src family members during hematopoietic differentiation. In particular, changes in the expression or activity of the src family members Hck, Lyn and Fgr have been found to be associated with myeloid differentiation (reviewed by Punt, 1992). The capacity of the murine homolog of Lsk/Hyl/Matk to phosphorylate Lck when coexpressed in yeast, together with the recent demonstration of Matk's ability to phosphorylate the C-terminal tyrosine of Src, suggests that this newest Csk-family will be intimately involved in the regulation of src- family kinases during hematopoiesis (Chow *et al.* 1994; Avraham *et al.*, 1995). Definitive proof of such a hypothesis may come from the study of animals in which the Lsk/Hyl/Matk locus is disrupted.

In contrast to the src family of protein tyrosine kinases, the regulation of Csk family members does not appear dependent on increases in catalytic activity. To date, only one group has reported modulation of Csk catalytic activity (Oetken *et al.*, 1994), while Lsk/ Hyl/Matk enzymatic regulation has not been documented. Interestingly, in contrast to Lsk/Hyl/Matk, the levels of Csk expression are largely unchanged after treatment with a variety of stimuli. These data suggest that in contrast to Csk, translational control is one means of Lsk/Hyl/Matk regulation. In fact, recent reports have suggested that translocation of Csk to the plasma membrane may be a primary means of regulating the interaction of this PTK with src family members and/or structures involved in adherence (Howell & Cooper, 1994; Bergman *et al.*, 1995). Consistent with this, stimulation of the Fc-receptor results in translocation of Csk and association with a 36 kDa tyrosine phosphorylated integral membrane protein that appears to be a Csk substrate (Ford *et al.*, 1994). In addition, artificial targeting of Csk to the membrane produces more dramatic inhibition of T cell receptor (TCR) function, apparently through activity downregulation of TCR-associated src family members such as Fyn (Chow *et al.*, 1993). Considered in total, it appears that in direct contrast to Csk, regulation of Lsk/Hyl/Matk protein levels may reflect a primary mechanism through which this PTK modulates signal transduction, however, potential regulation through stimuli- induced translocation is presently being studied in our laboratory.

In conclusion, our studies demonstrate that Lsk, Matk, and Hyl likely encode the same Csk- family PTK. Based on the structural similarity of Lsk/Matk/ Hyl to Csk and the capacity of both Matk, and the murine homologs to phosphorylate the src-family protein, Lck, on the carboxyl terminus in vitro (Chow *et al.*, 1994b; Avraham *et al.*, 1995), we and the other original authors now propose this PTK to be termed Chk (Csk homologous kinase). Our studies show striking regulation of Chk (Lsk/Matk/Hyl) mRNA and protein levels in response to the hematopoietic growth factor SCF. Further, PMA, an agent which can induce platelet markers in megakaryocytic cell lines, also induced Chk (Lsk/Matk/ Hyl) expression. In contrast to Chk (Lsk/Matk/ Hyl), no examined stimuli induced

significant increases in the more ubiquitously expressed Csk. Together with our previous findings, these studies suggest that Chk (Lsk/Matk/Hyl) may play a role in the signal transduction pathways common to SCF, PMA, IL-4 and IL-13 and demonstrate differential regulation of Csk family members in response to multiple cytokines.

Acknowledgements

The authors would like to thank Dr. Howard Young for the beta actin cDNA, Dr. Hava Avraham for the Matk cDNA and for the Sald03 antibody and Dr. Marietta Harrison for the anti-Csk antibody. In addition we thank Susan Charbonneau and Joyce Vincent for secretarial and editorial support. The publisher or recipient acknowledges right of the U.S. Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article.

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