9. Myocytes were whole-cell voltage-clamped with 1.5- to 2.5-megohm pipettes containing 110 mM CsCl, 10 mM NaCl, 5 mM magnesium adenosine triphosphate, 10 mM Hepes buffer, 20 mM tetraethylammonium chloride, 0.01 mM adenosine 3',5'-monophosphate, and 0.2 mM potassium salt of fura-2. External Tyrode's solution contained 137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, and 2 mM $CaCl_2$ at pH 7.4. K^+ in the external solution was replaced with Na^+ after the cells were perfused and equilibrated for 5 min. Cells were perfused for at least 10 min before test protocols were performed. All experiments were performed at room temperature (20° to 23°C).
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20. The fuzzy space model is based on several assumptions: (i) the Na^+ current has to be large—for example, 50 nA as suggested previously (7), (ii) a diffusion barrier for Na^+ and Ca^{2+} has to exist to delimit a subsarcolemmal space, (iii) the Na^+-Ca^{2+} exchanger has to operate at a sufficiently fast rate to bring enough Ca^{2+} into the space, and (iv) all molecular entities including Na^+ channels, Ca^{2+} channels, Na^+-Ca^{2+} exchanger, and SR release channels have to be located close together to interact intimately. Some of these assumptions remain untested.
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Tyrosyl Phosphorylation and Activation of MAP Kinases by p56^{lck}

ELHAM ETTEHADIEH, JASBINDER S. SANGHERA, STEVEN L. PELECH, DENISE HESS-BIENZ, JULIAN WATTS, NILABH SHASTRI, RUEDI AEBERSOLD*

T cell signaling via the CD4 surface antigen is mediated by the associated tyrosyl protein kinase p56^{lck}. The 42-kilodalton mitogen-activated protein (MAP) kinase (p42^{mapk}) was tyrosyl-phosphorylated and activated after treatment of the murine T lymphoma cell line 171CD4+, which expresses CD4, with antibody to CD3. Treatment of the CD4-deficient cell line 171 with the same antibody did not result in phosphorylation or activation of p42^{mapk}. Purified p56^{lck} both tyrosyl-phosphorylated and stimulated the seryl-threonyl phosphotransferase activity of purified p44^{mpk}, a MAP kinase isoform from sea star oocytes. A synthetic peptide modeled after the putative regulatory phosphorylation site in murine p42^{mapk} (Tyr^{185}) was phosphorylated by p56^{lck} with a similar V_{max}, but a fivefold lower Michaelis constant (K_m) than a peptide containing the Tyr^{394} autophosphorylation site from p56^{lck}. MAP kinases may participate in protein kinase cascades that link Src family protein-tyrosyl kinases to seryl-threonyl kinases such as those encoded by *rsk* and *raf*, which are putative substrates of MAP kinases.

MEMBERS OF A FAMILY OF TYrosyl-phosphorylated 42- to 45-kD seryl-threonyl kinases, known both as mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinases (ERKs), have been implicated in a variety of cytokine signal transduction pathways and in cell cycle control (*1*). Both p42^{mapk} (*2*) and p43^{erk1} (*3*) exhibit increased myelin basic protein (MBP) phosphotransferase activity after stimulation of quiescent mammalian cells with insulin and other mitogens. The p44^{mpk} is activated near the onset of germinal vesicle breakdown at M phase in maturing sea star oocytes (*4*). Enzymological and immunological characterization of p42^{mapk}, p43^{erk1}, and p44^{mpk} supports their relatedness (*3, 5*), and primary sequence analysis has revealed at least 80% amino acid identity between these isoforms (*6*). Tyrosyl phosphorylation is required for activation of each of these MAP kinases (*3, 7, 8*), but the responsible tyrosyl kinases have not been identified.

Stimulation of Jurkat cells and normal human T cells with antibodies to CD3 and phorbol ester tumor promoters such as phorbol myristate acetate (PMA) results in tyrosyl phosphorylation of two proteins of 42 and 43 kD, which were tentatively identified as MAP kinases (*9*). CD4 is a cell surface glycoprotein that recognizes nonpolymorphic regions of class II antigens of the major histocompatibility complex and potentiates the signal induced by stimulation of the CD3–T cell antigen receptor complex. A fraction of CD4 protein directly interacts with the CD3–T cell receptor complex (*10*). The functional importance of CD4-CD3–T cell receptor interaction is further supported by the observation that coaggregation of CD3 and CD4 with monoclonal antibodies enhances CD3-mediated activation of MAP kinase in Jurkat cells, although antibodies to CD4 alone failed to elicit stimulation of MAP kinase activity (*9*). The tyrosyl kinase p56^{lck} is physically associated with the cytoplasmic tail of CD4 via cysteine motifs (*11*) and is activated when CD4 is cross-linked with monoclonal antibodies (*12*). T cell lines expressing CD4 protein with altered or truncated cytoplasmic tails are defective in CD4 signal transduction (*13, 14*).

Mono Q ion-exchange chromatography of cytosol from the murine T lymphoma cell line 171CD4+ (*13*) resolved a peak of MBP phosphotransferase activity that was selectively stimulated after treatment of the cells for 5 min with monoclonal antibody 2C11 to CD3 (anti-CD3) (Fig. 1A). When the Mono Q fractions surrounding this peak were probed on protein immunoblots with monoclonal antibody 4G10 to phosphotyrosine, an immunoreactive protein of 42 kD that cofractionated with the activated MBP kinase was evident only with extracts from cells treated with anti-CD3 (Fig. 1, B and C). A 44-kD phosphotyrosine-containing protein was detected in extracts from both untreated cells and those exposed to anti-CD3. Identification of the 42- and 44-kD phosphoproteins as MAP kinases was supported by their immunoreactivity with affinity-purified rabbit polyclonal antibodies (anti-Erk1-CT) to a synthetic peptide patterned after the COOH-terminal 35 residues of rat p43^{erk1} (*6*). Anti-Erk1-CT cross-reacted with purified preparations of sea star p44^{mpk}, murine p42^{mapk}, and human p43^{erk1} (*15*). In Mono Q fractions from both control and anti-CD3–stimulated cells, anti-Erk1-CT reacted intensely with protein bands of 41 and 44 kD (Fig. 1, D and E). However, after treatment of cells with anti-CD3, proteins of 42 and 45 kD were also detected. Such band shifts on SDS-polyacrylamide gels are typical consequences of protein phosphorylation. The simplest interpretation of these findings is that CD3-mediated stimulation of the T cells induced tyrosyl phosphorylation and activation of p42^{mapk}, but a 44-kD MAP kinase was already tyrosyl-phosphorylated in un-

E. Ettehadieh, J. S. Sanghera, S. L. Pelech, D. Hess-Bienz, J. Watts, R. Aebersold, The Biomedical Research Centre and the Departments of Biochemistry and Medicine, University of British Columbia, Vancouver, B.C. V6T 1Z3 Canada.
N. Shastri, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

*To whom correspondence should be addressed.

treated cells. This latter MAP kinase is distinct from $p44^{mpk}$, but probably corresponds to murine $p43^{erk1}$.

To confirm that the increase in MBP kinase activity observed on Mono Q corresponded to activation of $p42^{mapk}$, extracts from anti-CD3–stimulated and unstimulated 171CD4+ cells were fractionated on phenyl-Superose. This resin binds $p42^{mapk}$ more avidly than the 44-kD MAP kinase (*5*). Whereas the MBP phosphotransferase activity of the 44-kD MAP kinase was unchanged after stimulation with anti-CD3, the activity of $p42^{mapk}$ was increased fivefold with a corresponding enhancement in tyrosyl phosphorylation (*15*).

To investigate whether $p56^{lck}$ participates in anti-CD3–induced tyrosyl phosphorylation and activation of $p42^{mapk}$, we used the cell line 171, which does not express CD4 (171CD4−) and is uncoupled from the $p56^{lck}$-dependent activation pathway (*13*). Protein immunoblot analysis of the cytosolic extracts from 171CD4− cells indicated that anti-CD3 failed to induce tyrosyl phosphorylation of the 42-kD protein that was most

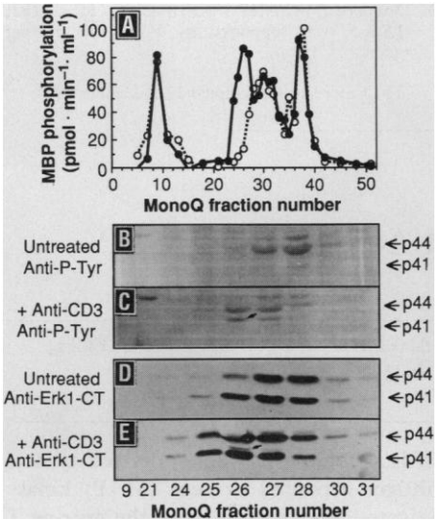

Fig. 1. CD3-mediated activation of MAP kinase in murine T cells. The murine T cell line 171CD4+ (*13*) was incubated at 37°C for 5 min in 30 mm plastic dishes coated without (○) or with (●) the 2C11 monoclonal antibody to CD3 before sonication of the cells for 20 s with a Vibra Cell sonicator (setting 40) in 0.5 ml of homogenization buffer [50 mM tris-HCl (pH 7.5), Nonidet P-40 (1%), 150 mM NaCl, 5 mM EDTA, leupeptin (0.5 μg/ml), aprotinin (10 μg/ml), pepstatin (0.7 μg/ml), soybean trypsin inhibitor (10 μg/ml), 0.2 mM sodium orthovanadate, 10 mM NaF, and 1 mM sodium molybdate]. The homogenate was centrifuged at 14,000 rpm for 2 min at 4°C in an Eppendorf microfuge and the supernatant was stored at −70°C. Detergent-solubilized supernatant protein [1 mg from untreated and 0.75 mg from anti-CD3 treated cells as determined by the Bradford method (*16*)] was loaded onto a Mono Q fast protein liquid chromatography column (Pharmacia) in buffer A [5 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.2), 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 0.5 mM NaF] and eluted with a 10-ml linear gradient of 0 to 0.8 M NaCl in buffer A. (**A**) Column fractions (0.25 ml) were assayed for phosphotransferase activity toward MBP (1 mg/ml) as described (*8*), and the data were normalized for the application of 1 mg of protein to the column. Portions (100 μl) of selected Mono Q fractions from untreated (**B** and **D**) and anti-CD3–treated (**C** and **E**) cells were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (*17*), and the resolved proteins were transferred onto PVDF membranes. The membranes were immunoblotted as described (*8*) with the 4G10 murine monoclonal antibody to phosphotyrosine (anti-P-Tyr) (U.B.I.) (B and C) and affinity-purified rabbit polyclonal antibodies (anti-Erk1-CT) (U.B.I.) to a synthetic peptide patterned after the COOH-terminal 35 residues of rat $p43^{erk1}$ (*6*) (D and E). The top and bottom of each panel in this figure and in Fig. 2 correspond to the electrophoretic migrations of the prestained marker proteins ovalbumin (50 kD) and carbonic anhydrase (33 kD), respectively; the position of tyrosyl-phosphorylated $p42^{mapk}$ is indicated with an arrow.

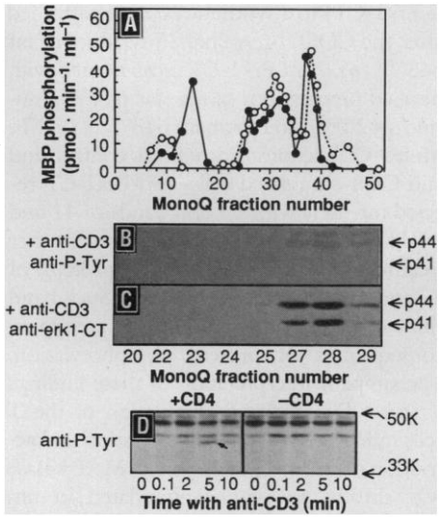

Fig. 2. Lack of CD3-mediated activation of MAP kinase in CD4-deficient cells. The murine T cell line 171CD4− (*13*) was incubated for 5 min at 37°C in 30-mm plastic dishes coated without (○) or with (●) the 2C11 antibody to CD3. Detergent-solubilized supernatant fractions prepared from these cells were subjected to Mono Q chromatography and assayed for MBP phosphotransferase activity (**A**) as described (Fig. 1). Portions (100 μl) of selected Mono Q fractions from anti-CD3–treated cells were subjected to protein immunoblotting analysis with anti-P-Tyr (**B**) and anti-Erk1-CT (**C**). Cytosolic protein (0.5 mg) from 171CD4+ (+CD4) and 171CD4− (−CD4) cells that had been incubated for 0 to 10 min with anti-CD3 was analyzed by the protein immunoblotting procedure with anti-P-Tyr (**D**).

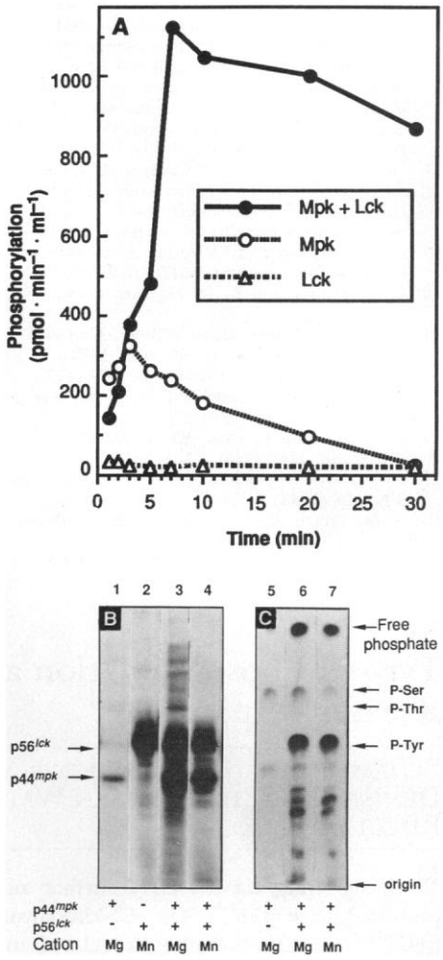

Fig. 3. In vitro phosphorylation and activation of $p44^{mpk}$ by $p56^{lck}$. (**A**) Purified sea star $p44^{mpk}$ (1 μg/ml) was incubated at 30°C for 0 to 30 min in the absence (○) and presence (●) of purified murine $p56^{lck}$ (0.5 μg/ml) with 20 μM ATP and 5 mM $MnCl_2$, and subsequently assayed for 10 min for phosphotransferase activity towards the synthetic peptide APRTPGGRR (2 mM) with 250 μM [γ-^{32}P]ATP in buffer B [20 mM MOPS (pH 7.2), 10 mM $MgCl_2$, 1 mM sodium orthovanadate, and 1 mM dithiothreitol] as described (*19*). In control experiments, $p56^{lck}$ was incubated and assayed for APRTPGGRR phosphotransferase activity in the absence of $p44^{mpk}$ (△). (**B**) Purified $p56^{lck}$ (60 ng) and $p44^{mpk}$ (400 ng) were incubated at 30°C for 20 min in the presence of 10 mM $MgCl_2$ (lane 3) or 10 mM $MnCl_2$ (lane 4) with 20 μM [γ-^{32}P]ATP in buffer C [25 mM tris-HCl (pH 7.5) and 1 mM dithiothreitol]. The kinases $p44^{mpk}$ (lane 1) and $p56^{lck}$ (lane 2) were also incubated separately with 20 μM [γ-^{32}P]ATP in buffer C with 10 mM $MgCl_2$ and 10 mM $MnCl_2$, respectively. Reactions were terminated with SDS-polyacrylamide gel sample buffer and the proteins were separated by SDS-PAGE (*17*); the resolved proteins were transferred to a PVDF membrane. An autoradiogram of the PVDF membrane is shown. Lane 1 corresponds to a threefold longer exposure of the x-ray film than that shown in lanes 2 to 4. (**C**) The $p44^{mpk}$ bands shown in (B) (lanes 1, 3, and 4) were excised from the membrane and subjected to phosphoamino acid analysis (*5*). An autoradiogram of the thin-layer chromatography plate is shown, and the migrations of the standard phosphoamino acids are indicated.

evident in extracts from 171CD4+ cells treated for 5 min with the same antibody (Fig. 2D). Antibodies to phosphotyrosine did not detect the 42-kD protein after Mono Q fractionation of the cytosol from 171CD4− cells that had been incubated for 5 min with anti-CD3 (Fig. 2B), nor was a stimulated peak of MBP phosphotransferase activity detected (Fig. 2A). However, the 171CD4− cells still had normal amounts of dephosphorylated 41-kD ($p42^{mapk}$) and 44-kD MAP kinases as detected by anti-Erk1-CT (Fig. 2C). These results demonstrate CD4-dependent regulation of MAP kinases in vivo through a pathway requiring $p56^{lck}$. These results also confirm that CD3-T cell receptor and CD4 proteins form functional receptor conglomerates on the surface of T cells (*10*).

To further investigate the regulation of MAP kinase activity by tyrosyl phosphorylation by $p56^{lck}$, $p44^{mpk}$ was tested as a substrate in vitro. Recombinant murine $p56^{lck}$ was purified from baculovirus-infected sf9 cells (*18*), and $p44^{mpk}$ was isolated from maturing sea star oocytes (*5*). Incubation of a relatively inactive preparation of $p44^{mpk}$ with active $p56^{lck}$ in the presence of Mg^{2+} or Mn^{2+} increased incorporation of ^{32}P from [γ-^{32}P]ATP (adenosine triphosphate) into $p44^{mpk}$ in comparison to that detected in the absence of $p56^{lck}$ (Fig. 3B). The stoichiometry of phosphorylation of $p44^{mpk}$ by $p56^{lck}$ was approximately 0.25 mole of phosphate per mole of $p44^{mpk}$. Moreover, incubation of $p56^{lck}$ with $p44^{mpk}$ led to a time-dependent activation of the phosphotransferase activity of the MAP kinase (Fig. 3A). A synthetic peptide substrate, APRTPGGRR (*19*), corresponding to the Thr^{97} phosphorylation site in MBP, which does not contain a tyrosyl residue, was used for this experiment because MBP is also a substrate for $p56^{lck}$ (*18*). A tenfold activation of $p44^{mpk}$ was achieved after a 10-min incubation with $p56^{lck}$.

Increased tyrosyl phosphorylation of $p44^{mpk}$ by $p56^{lck}$ could not be unequivocally shown by immunoreactivity with anti-P-Tyr because purified $p44^{mpk}$ is already partially phosphorylated on tyrosyl residues (*8*). Purified $p44^{mpk}$ autophosphorylates on seryl residues upon incubation with [γ-^{32}P]ATP in vitro; autophosphorylation results in partial activation of the kinase (*8*). The ^{32}P-labeled $p44^{mpk}$ phosphorylated by $p56^{lck}$ was subjected to phosphoamino acid analysis. Tyrosyl phosphorylation of $p44^{mpk}$ was increased after incubation with active $p56^{lck}$ (Fig. 3C) but not in a control experiment in which $p56^{lck}$ was first inactivated by heat treatment (*15*). Because $p44^{mpk}$ was already partially tyrosyl-phosphorylated, the incorporation of 0.25 mole of ^{32}P per mole, predominantly on tyrosine, represented a relatively high degree of phosphorylation.

Thr^{183} and Tyr^{185} are the regulatory sites for activation of $p42^{mapk}$ (*20*). These and the surrounding residues are highly conserved in MAP kinases, but the residue equivalent to murine $p42^{mapk}$ Thr^{183} in sea star $p44^{mpk}$ appears to be substituted (*6*). These phosphorylation sites are located just before the conserved protein kinase subdomain VIII (*21*), which is implicated in ATP binding. To evaluate whether $p56^{lck}$ recognized such a phosphorylation site, we used a synthetic peptide (ADPDHDHTGFLTEYVATRWRR) that encompassed the putative Tyr^{185} phosphorylation site in murine $p42^{mapk}$. This peptide was phosphorylated by purified $p56^{lck}$ in vitro with an apparent Michaelis constant (K_m) of ~170 μM and a V_{max} of 90 pmol min^{-1} per microgram of protein. It was a better substrate for $p56^{lck}$ than a peptide (KRLIEDEYTARQGGC) patterned after the putative Tyr^{394} autophosphorylation site in $p56^{lck}$ (apparent K_m ≃ 900 μM; V_{max} = 80 pmol min^{-1} per microgram of protein). Because $p56^{lck}$ activated $p44^{mpk}$ and it could phosphorylate a peptide that resembled the putative regulatory tyrosyl phosphorylation site in $p42^{mapk}$, we conclude that $p56^{lck}$ probably phosphorylated $p42^{mapk}$ at this site in the 171CD4+ cells stimulated with anti-CD3.

It has been ambiguous whether MAP kinases are phosphorylated by another protein-tyrosyl kinase or are stimulated to undergo tyrosyl autophosphorylation (*6, 22–24*). We have demonstrated that a MAP kinase is tyrosyl-phosphorylated and activated by the tyrosyl kinase $p56^{lck}$. It seems likely that MAP kinases may also be directly regulated by other members of the Src-like protein-tyrosyl kinase family. Some of the putative targets of MAP kinases are other protein kinases such as the ribosomal S6 kinases encoded by the *rsk* gene family (*25*) and the *raf* gene product (*26*). Thus, MAP kinases may take part in cascades of sequentially activated protein kinases.

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