Ultraviolet Light-induced Stimulation of the JNK Mitogen-activated Protein Kinase in the Absence of Src Family Tyrosine Kinase Activation

Received for publication, March 27, 2000, and in revised form, April 27, 2000
Published, JBC Papers in Press, May 8, 2000, DOI 10.1074/jbc.M002573200

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In T cells, the JNK mitogen-activated protein kinase is activated by simultaneous stimulation of the T-cell receptor and CD28 or by a number of stress stimuli including ultraviolet light, hydrogen peroxide, and anisomycin. Lck, a Src family kinase, is essential for T-cell receptor-mediated activation of JNK. We asked whether Lck was also involved in stress-mediated activation of JNK. JNK was activated by ultraviolet light irradiation in all of the four T-cell lines we examined, but Lck was not. Additionally, JNK activation by ultraviolet light, hydrogen peroxide, and anisomycin was completely normal in T cells lacking Lck. These data suggest that Lck is not activated by ultraviolet light irradiation, nor is it required for JNK activation in T cells by any of the stress stimuli we tested. We also examined JNK activation by ultraviolet light in mouse fibroblasts expressing no known Src kinases. The activation of JNK by ultraviolet light was completely normal in these cells. Finally, treatment of lymphoid and epithelial cells with a Src kinase family inhibitor PP2-reduced tyrosine phosphorylation of cellular proteins markedly without affecting ultraviolet light-induced activation of JNK. These results suggest that Src kinases are not essential for ultraviolet light-induced activation of JNK in a diverse variety of cell types.

The c-jun N-terminal kinases (JNK), also known as the stress-activated protein kinases, are members of the mitogen-activated protein kinase family (1–3). They are stimulated by exposure of cells to a diversity of stimuli including protein synthesis inhibitors such as anisomycin, oxidative agents such as hydrogen peroxide, genotoxins such as ultraviolet light (UV), and cytokines such as interleukin-1 (2–6). The c-jun N-terminal kinases are also stimulated during lymphocyte activation following stimulation of the T-cell receptor and CD28 (7). The JNK family kinases have a number of identified substrates including activated transcription factor-2 and c-jun (8, 9). Phosphorylation of serine 63 and serine 73 on c-jun by JNK is necessary for maximal c-jun transcriptional activity (1, 10). JNK1 and JNK2 are widely expressed in diverse cell types including lymphocytes, whereas JNK3 expression is restricted to brain neurons (8, 9, 11). JNK1 null mice or JNK2 null mice are viable. Both strains, however, exhibit decreased activation-induced T-cell death and an imbalance in TH1- and TH2-mediated immune responses (12–14). These data suggest that JNK1 and JNK2 are both important for regulation of cell viability and differentiation in T lymphocytes. Deletion of both JNK1 and JNK2 causes embryonic lethality due to severe disorganization of apoptosis during brain development (15).

In HeLa cells, UV irradiation has been reported to activate the c-Src tyrosine-protein kinase and based on the inhibitory effect of expressing catalytically inactive v-src, it has been concluded that Src activity is essential for UV-induced activation of JNK in these cells (16). However, neither the mechanism of Src activation by UV light nor how this leads to JNK stimulation is clear. It is likely that Lck, a Src kinase expressed predominantly in T lymphocytes (17), is involved in activation of JNK following ligation of the T-cell receptor and CD28 (18). Lck is required for T cell maturation (19, 20) and signaling from the T-cell antigen receptor in mature T lymphocytes. A derivative of the Jurkat T-cell line that has lost expression of full-length Lck, JCaM1, is unresponsive to stimulation through the T-cell receptor (20). These JCaM1 cells have greatly reduced calcium flux into the cytoplasm and JNK activation under conditions that fully stimulate their parental Jurkat T cells (18, 20). Because reintroduction of Lck restores signaling, calcium flux, and JNK activation, the absence of Lck is responsible for the signaling defect from the T cell receptor in these cells.

The mechanism for activation of JNK after UV exposure is not understood. UV-induced activation of JNK is not necessarily because of chromosomal DNA damage, because JNK stimulation is observed in enucleated cells (21). This suggests that there is at least one UV target other than DNA responsible for activation of JNK. A role for reactive oxygen intermediates in the propagation of the UV signal has been suggested. N-acetylcysteine, a glutathione precursor that elevates the reducing potential in the cytoplasm, inhibits UV-induced JNK activation and c-jun phosphorylation (16, 22–24). This suggests that UV irradiation may lead to elevated levels of oxidants that either directly or indirectly lead to JNK stimulation. Lck is activated by hydrogen peroxide (25–27), a progenitor of reactive oxygen and our assay measures c-jun N-terminal kinase activity, in this report JNK refers to both JNK1 and JNK2.

* This work was supported by Grants CA14195, CA17289, and CA42350 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: JNK, c-jun N-terminal kinase; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase; TNF, tumor necrosis factor; PAGE, polyacrylamide gel electrophoresis.

2 Because both JNK1 and JNK2 are comparably expressed in T cells...
intermediates. Because UV apparently generates reactive oxygen intermediates and has been reported to activate the c-Src kinase, it was reasonable to expect that ultraviolet light might also activate Lck in T cells. Furthermore, the finding that Lck is essential for stimulation of JNK and extracellular signal-regulated kinase mitogen-activated protein kinases after T-cell activation (18) suggested that Lck is essential for propagation of some upstream signals to these mitogen-activated protein kinases. We therefore asked whether and how UV irradiation activated Lck in T cells and whether Src family kinases were essential for UV-induced activation of JNK in a variety of cells including T lymphocytes.

EXPERIMENTAL PROCEDURES

Construction of Lck Mutants and Generation of Stable Cell Lines— Wild type and mutant Lck constructs were derived from murine lck cDNA (28). The construction of A2F505 Lck has been described (25, 29). 208F rat fibroblasts were infected with the retroviral expression vector LXS containing either wild type or A2F505 Lck as described (29).

Cell Lines—208F rat fibroblasts expressing wild type or A2F505 Lck were maintained in Dulbecco-Vogt modified Eagle’s medium (Media- tech) supplemented with 10% calf serum (Gemiini), and 600 μg/ml G418 (Geneticin). Jurkat (30), JCaM 1 (20), and HPB-MLT (31) cells, all human leukemia T-cell lines, were maintained in RPMI 1640 medium supplemented at 4 °C, 30 mM 32P-labeled [γ-32P]ATP for 1, 3, and 5 min at room temperature. The reactions were stopped by the addition of SDS-polyacrylamide gel loading sample buffer. Treatment of Cells With Hydrogen Peroxide, Pervanadate, Anisomycin, Ultrasound Light, and PP2—Adherent cells were seeded at 1 × 106 cells on 10-cm plates and allowed to recover for 24 h. They were subsequently starved in medium containing 0.25% calf serum for 16 h. For treatments with pervanadate, 1 mM hydrogen peroxide was added at 30 min, 100-fold molar excess of hydrogen peroxide at room temperature for 20 min. The residual hydrogen peroxide was degraded in a 20-min incubation with catalase (Sigma) at room temperature, and pervana- date was added at a final concentration of 200 μM. Anisomycin (Calbio- chem) was administered at a final concentration of 50 μg/ml. UV irra- diation was performed as described previously (3). Tissue culture medium was removed to abrogate absorption of UV light by the phenol red in the medium. Adherent cells were exposed directly on the plate to a calibrated UV light source (General Electric, two G15T8 15 W bulbs) that emitted 8.5 J/m2/s. The dose of UV irradiation was controlled by varying the length of exposure to the lamp. Unless indicated otherwise, cells were exposed to 40 J/m2 UV irradiation. After treatment with one of the aforementioned agents, the cells were incubated for 15 and 40 min at 37 °C for Lck and JNK assays, respectively. Cells were washed with ice-cold isotonic Tris-buffered saline and subsequently lyzed directly on the tissue culture plates. Suspension cells were counted and resuspended at 1 × 106 cells/ml in tissue culture medium containing 0.25% calf serum. Suspension cells were exposed to the same doses of each stimulus used for adherent cells. UV irradiation was performed in medium lacking phenol red. After treatment, suspension cells were centrifuged at 400 × g in a Beckman TJ-6 centrifuge for 5 min and washed once with chilled isotonic Tris-buffered saline before lysis. PP2 (Calbiochem) was used to specifically inhibit Src family tyrosine-protein kinases (37). Cells were pretreated with either 10 or 20 μM PP2 for 2 h before exposure to UV light. The cells were incubated for an additional 40 min after UV irradiation in the presence of PP2 before lysis.

RESULTS

Lck Activity Is Unaffected by Irradiation with Ultraviolet Light in Jurkat T Cells—We asked whether Lck activity was altered by UV irradiation in human Jurkat T cells. To confirm that these cells responded to UV irradiation, we assayed Lck and JNK activity in parallel. A GST-Jun chimera was used to bind kinases that interact with the N terminus of c-Jun, and the bound kinase was subsequently assayed in vitro for its ability to phosphorylate the GST-Jun protein. We confirmed that this method of measuring JNK activity gave similar results to im-munoprecipitation of epitope-tagged JNK1 and JNK2. Because both JNK1 and JNK2 are expressed in T cells and our assay does not distinguish between them, here the term JNK refers to both JNK1 and JNK2.

Irradiation of Jurkat T cells with 40 J/m2 UV light led to a 10-fold increase in the activity of JNK (Fig. 1A). Lck was unaffected by this dose of UV irradiation (Fig. 1B). In contrast, pervanadate (200 μM) stimulated both Lck and JNK 10-fold, and hydrogen peroxide (500 μM) activated Lck and JNK 5-10-fold, respectively (Fig. 1A and B). UV-induced activation of
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JNK was detectable in Jurkat cells after irradiation with as little as 20 J/m² ultraviolet light. JNK activity increased steadily in response to increasing doses of UV irradiation up to 1000 J/m², where 25-fold activation was seen, and declined gradually at higher doses (data not shown). In contrast, Lck activity was not changed detectably at any dosage of UV irradiation up to 1200 J/m², a level of irradiation 60-fold higher than that required to activate JNK (Fig. 1E). Optimum activation of JNK occurs 40 min after UV irradiation. Lck activation due to a stress stimulus such as hydrogen peroxide peaks 15 min after stimulation (25). We considered the possibility that Lck was in fact activated by UV irradiation for a brief period and that its activity had returned to basal levels by the time we measured it. Therefore, we assayed Lck activity at 0, 2, 5, 15, and 30 min after 200 J/m² UV irradiation. We found no detectable change in Lck activity at any time following UV stimulation (Fig. 1F).

To determine whether the lack of UV-induced activation of Lck activity in T cells was peculiar to Jurkat cells, we asked whether UV activated Lck in mouse D011.10 hybridoma T cells, human HPB-MLT leukemic T cells, and mouse CTLL-2 T cells. UV irradiation activated JNK 8–10-fold in all three cell lines but had no detectable effect on Lck activity in any of the cell lines (data not shown). In contrast, pervanadate activated both JNK and Lck in all three cell lines 8–10- and 3–5-fold, respectively.

**Lck Activity in 208F Rat Fibroblasts Is Insensitive to Ultraviolet Light—Activation of c-Src by UV irradiation has been observed in adherent cells (16). Although we could not detect UV-induced Lck activation in T cells, we asked whether more robust UV-induced activation of Lck might occur when Lck was expressed in adherent cells. We therefore examined the effect of UV irradiation on the activity of Lck expressed stably in rat 208F fibroblasts. 40 J/m² ultraviolet light induced an 8–10-fold increase in JNK activity in these cells (Fig. 2A). Lck activity was unaffected under the same conditions (Fig. 2C). To confirm that we had not missed a brief period of Lck activation, we examined Lck activity at 0, 2, 5, 15, and 30 min after 200 J/m² UV irradiation. Lck activity in adherent cells remained unchanged at all time points after stimulation as we had observed in Jurkat T cells (data not shown).

It has been postulated that the UV signal is propagated by a reactive oxygen species (16, 22). We have shown previously that the activity of A2F505 Lck, a cytosolic Lck mutant, is noticeably more sensitive to oxidants than wild type Lck. Hydrogen peroxide, which stimulates wild type Lck 3–5-fold, activates A2F505 Lck over 10-fold. These results indicate that neither wild type nor A2F505 Lck are activated by UV light irradiation in 208F fibroblasts expressing A2F505 Lck but did not alter the activity of A2F505 Lck detectably (Fig. 2, A and D). In contrast, hydrogen peroxide activated both A2F505 Lck and JNK over 10-fold. These results indicate that neither wild type nor A2F505 Lck are activated by UV light irradiation in 208F fibroblasts under conditions that stimulate JNK dramatically.

**Ultraviolet Light-induced Activation of JNK in T Cells Is Not Dependent on Lck**—JNK is stimulated in T cells after activation of T cells with UV light. However, it is not clear whether this activation is dependent on Lck. We therefore asked whether it was possible that UV irradiation activated JNK in T cells in the absence of Lck. To test this hypothesis, we examined Lck activity in T cells from Lck-/- mice. We found that UV irradiation activated JNK 8–10-fold in Lck-/- T cells, similar to what we observed in wild type T cells (data not shown). These results indicate that UV irradiation activates JNK in T cells in the absence of Lck and suggest that UV irradiation activates JNK in T cells through a mechanism independent of Lck.

GST-Jun quantities in each sample were determined by staining with Coomassie Brilliant Blue. F Lck immunoprecipitates were prepared 15 min after irradiation of Jurkat T cells with the indicated dose of ultraviolet light, 200 μM pervanadate (PV), or mock irradiation. F, lysates for the analysis of Lck activity were prepared at the indicated time points (min) after 200 J/m² UV irradiation. Mock-irradiated cells were lysed 30 min after sham irradiation, and cells stimulated with 500 μM hydrogen peroxide (H₂O₂) were lysed after 15 min of exposure.

**FIG. 1. Effect of UV, H₂O₂, and pervanadate on activity of Lck and JNK in Jurkat T cells.** For A–D, Jurkat T cells were exposed to either 40 J/m² ultraviolet light (UV), 500 μM hydrogen peroxide (H₂O₂), 200 μM pervanadate (PV), or mock irradiation. A, lysates for the analysis of LNK activity were prepared 40 min after stimulation and assayed in vitro for their ability to phosphorylate GST-Jun. The samples were fractionated by SDS-PAGE. Incorporation of ³²P into GST-Jun was quantified on a PhosphorImager and normalized to the amount of Lck detectably (25). B, GST-Jun substrate in each sample was quantified on a PhosphorImager and normalized to the amount of Lck detectably (25). C, for normalization, Lck protein levels in the samples were measured by Western blotting of a fraction of the immunoprecipitates with antibody to Lck and ¹²⁵I-protein A. D.
tion induced by cross-linking with anti-CD3 and anti-CD28 antibodies (7). It has been shown that this CD3/CD28-dependent activation of JNK in T cells is Lck-dependent (18). Although Lck was not activated detectably by UV light in T cells, we asked whether Lck was required for UV-induced activation of JNK. To this end we utilized JCaM.1 cells, a derivative of Jurkat T cells lacking functional Lck (20). JCaM.1 cells are largely unresponsive to T-cell receptor stimulation. JNK activity in JCaM.1 cells was increased over 25-fold by UV irradiation (Fig. 3A). Treatment of both JCaM.1 and Jurkat cells with anti-CD3/CD28 antibodies (7). It has been shown that UV activation of JNK is normal in mouse embryonic fibroblasts, was not detectable in S F2 cells (33). We additionally examined UV-induced activation of JNK in triple knockout cells (S F2 F y F, S F2 F y F, and S F2 F y F). Lysates for the analysis of JNK activity were prepared, normalized, and presented as described in the legend to Fig. 1.

**Fig. 2.** Effect of UV and H2O2 on activity of wild type Lck, A2F505 Lck, and JNK in 208F fibroblasts. Cells expressing wild type (A and C) or A2F505 Lck (B and D) were exposed to either 40 J/m² ultraviolet light (UV), 50 μM hydrogen peroxide (H2O2), or mock irradiation. Lysates for the analysis of JNK activity were prepared 40 min after stimulation and assayed in vitro for their ability to phosphorylate GST-Jun. The samples were fractionated by SDS-PAGE. The amount of GST-Jun in each sample was determined by staining with Coomassie Blue. Incorporation of 32P into GST-Jun was quantified on a PhosphorImager and normalized to the amount of GST-Jun substrate in each sample (A and B). Lck immunoprecipitates were prepared 15 min after stimulation and assayed in vitro for their ability to phosphorylate Val1-angiotensin II. Assay results are expressed as the rate of labeled phosphate incorporation by the substrate/arbitrary unit of Lck (C and D).

**Fig. 3.** JNK activity in JCaM.1 and Jurkat cells. JCaM.1 (A) and Jurkat (B) cells were treated with either 40 J/m² ultraviolet light (UV), 50 μg/ml of anisomycin (Aniso), 200 μM pervanadate (PV), 500 μM hydrogen peroxide (H2O2), or mock irradiation. Lysates for the analysis of JNK activity were prepared, normalized, and presented as described in the legend to Fig. 1.

**JNK Activity Is Unaffected by Treatment with the Src Kinase Inhibitor PP2**—The pyrazolopyrimidine PP2 is a fairly specific inhibitor of Src family kinases. It inhibits the in vitro activity of Lck and Fyn kinases with an IC50 of 5 nM (37). In contrast, the IC50 of PP2 for ZAP-70 and JAK2, two tyrosine kinases expressed in hematopoetic cells that are not Src family members, is greater than 100 and 50 μM, respectively (37). As another approach for evaluating a role for Src kinases in UV-induced activation of JNK, we asked whether PP2 suppressed UV activation of JNK in 293T, Jurkat, and HeLa cells. A 2-h PP2 (20 μM) treatment of 293T cells ectopically expressing a genetically activated Lck (F505) reduced tyrosine phosphoryl-

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3. K. Amdjadi and B. M. Sefton, unpublished results.
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Fig. 4. JNK activity in mouse embryonic fibroblasts lacking Src kinases. Triple knock out S^Y^F^ cells, double knock out S^Y^F^-cells derived from a littermate embryo, and S^Y^F^-cells expressing c-Src from a stably introduced c-Src expression vector were treated with either 40 J/m^2^ ultraviolet light (UV), 200 mM pervanadate (PV), or mock irradiation. Lysates were prepared 40 min after stimulation and were assayed in vitro for their ability to phosphorylate GST-jun. The samples were fractionated by SDS-PAGE, stained with Coomassie Blue, and quantified on a PhosphorImager.

Fig. 5. JNK activity in 293T cells treated with the Src kinase inhibitor PP2. 293T cells expressing activated F505 Lck were pretreated with either 10 or 20 mM PP2 for 2 h and then exposed to either 40 J/m^2^ ultraviolet light (UV) or mock irradiation. Lysates were prepared 40 min after stimulation with continuous PP2 treatment. A, clarified lysates were fractionated by SDS-PAGE stained with antiphosphotyrosine rabbit polyclonal antibody and ^125^I-protein A. B, lysates for the analysis of JNK activity were assayedin vitro for their ability to phosphorylate GST-jun. The samples were fractionated by SDS-PAGE, stained with Coomassie Blue, and quantified on a PhosphorImager.

DISCUSSION

Exposure of bacteria to UV light and other DNA damaging agents induces the SOS response (38, 39). Either DNA damage or by-products of DNA damage are thought to initiate this response. The mammalian stress response was also originally attributed to DNA damage. However, recent work has shown that stress responses can occur in the absence of DNA damage (40–43) and that not all DNA damaging agents yield identical responses or produce the same outcome (40, 44). The stress response to reactive oxygen intermediates and UV light is likely to be triggered in the cytoplasm or the plasma membrane, because both nuclear factor-xB and activator protein-1 are activated by UV light in enucleated cells (21). It has been reported that UV irradiation activates the c-Src protein kinase in HeLa cells and UV-induced activation of JNK is inhibited by expression of a catalytically inactive v-Src protein (16). This suggested that c-Src was an essential component of the cellular response to ultraviolet light. Here, we asked whether UV-induced activation of Src kinases occurred in lymphocytes, and if so, was it important. Because Lck is an abundant member of the Src family of tyrosine-protein kinases in T lymphocytes, is activated readily by hydrogen peroxide, and is known to be essential for CD3/CD28-dependent activation of JNK, we examined the role of Lck in UV-induced activation of JNK in T cells (18, 25, 28, 45).

Exposure of either human or murine T cells to UV light did not stimulate Lck under conditions that activated JNK robustly (Fig. 1). We then asked whether UV-induced activation of Src kinases was unique to adherent cells. However, neither wild type Lck nor the hypersensitive A2F505 mutant of Lck were stimulated after UV irradiation of fibroblasts in which these Lck proteins were expressed ectopically (Fig. 2). Although Lck was not activated by UV irradiation, the possibility remained that Lck was required for the stimulation of JNK in an activation-independent manner. We therefore asked whether UV could activate JNK in JCaM.1 cells that lack Lck. We found that JNK was activated to the same extent in the

Lck-deficient JCaM.1 cells as in the parental Jurkat T cells (Fig. 3). Fyn, another Src kinase, was also unaffected by exposure to UV light in JCaM.1 and Jurkat cells, indicating that another abundant Src kinase was not compensating for the loss of Lck (data not shown).

We also examined the ability of UV to activate JNK in fibroblasts that express no known Src kinases (33). We found that UV-induced activation of JNK was intact in S^Y^F^-cells that express no Src kinases and indistinguishable from that observed in cells from littermates with functional c-src gene (Fig. 4). We finally evaluated the role of the Src family kinases in the UV response using the Src kinase inhibitor PP2. Under conditions where PP2 treatment inhibited more than 90% of total tyrosine phosphorylation, UV-induced activation of JNK in 293T cells ectopically expressing activated Lck remained largely intact (Fig. 5). PP2 treatment similarly reduced tyrosine phosphorylation in Jurkat T cells and human HeLa cells without noticeably affecting UV-induced activation of JNK (data not shown).

We conclude that the activity of Src kinases is not required for stimulation of JNK by UV irradiation in any of the cells we tested. Our results do not, however, suggest that Src kinases or tyrosine-protein kinases cannot activate JNK. Sodium vanadate, which induces elevated levels of tyrosine protein phosphorylation, activates JNK in a variety of cell types, and overexpression of Lck stimulates JNK in Jurkat T cells (18), rather our data suggest that in most cells Src kinases are not necessarily involved in, or required for, UV-induced activation of JNK. HeLa cells, in which c-Src has been reported to play an
important role in UV signaling, appear to be an exceptional cell line in this respect (16). We however observed that treatment of HiLa cells with the Src kinase inhibitor PP2 did not affect UV-induced activation of JNK (data not shown). It is possible that the observed inhibitory effect of inactive v-Src was due to general toxicity from overexpression of a catalytically inactive Src molecule that is unburdened by intramolecular regulation. Interestingly, NIH3T3 cells lacking the c-src gene exhibit reduced JNK activation by the alkylation agent methyl methanesulfonate but have normal UV-induced activation of JNK (46).

The apparent dispensability for Src kinases in activation of JNK is certainly not unprecedented. Stimulation of the Fas/ APO-1/CD95 receptor, a member of the tumor necrosis factor (TNF)-a receptor family (47), results in JNK activation and apoptosis in a wide variety of cell types (48). JNK activation by Fas stimulation is, however, normal in Lck-deficient cells, suggesting that Fas-induced activation of JNK is not Lck-dependent (49). TNF-a treatment results in clustering of the ubiquitously expressed p55 TNF-a receptor and subsequent stimulation of JNK in a wide variety of cell types (7, 48, 50, 51). Irradiation of cells with UV is also reported to lead to the clustering of TNF-a receptors (52). This clustering may be in fact critical in UV-induced activation of JNK because irradiation with UV at 10 °C, below the transition temperature of the TNF-a receptor and subsequent clustering and JNK activation (52). The TNF-a receptor-associated death domain protein and TNF-a receptor-associated factor bind to the clustered TNF-a receptors (53–55) and mediate signaling to JNK (56–59). UV-induced activation of JNK may be dependent on triggering of the TNF-a receptor and signaling through TNF-a receptor-associated death domain protein and TNF-a receptor-associated factor proteins, whereas Lck and other Src kinases may be involved in activation of JNK only through the pathway downstream of the T-cell receptor and CD28.

Acknowledgments—We thank Estella Jacinto for providing numerous JNK reagents, and we are indebted to Richard Klinkhoff and Phillippe Soriano for the very generous gift of the Src molecule that is unburdened by intramolecular regulation. We are indebted to Richard Klinghoffer and Marc Sauvageau for the very generous gift of the Src molecule that is unburdened by intramolecular regulation. We are indebted to Richard Klinghoffer and Marc Sauvageau for the very generous gift of the Src molecule that is unburdened by intramolecular regulation.

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