Brief Definitive Report

Interleukin 2 Activates Extracellular Signal-regulated Protein Kinase 2

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Summary

Interleukin 2 (IL-2) stimulated activation of the 42-kD extracellular signal–regulated kinase 2 (Erk2) in murine IL-3–dependent cells, expressing either high or intermediate affinity IL-2 receptors. Activation was both rapid, occurring within 5 min of IL-2 addition, and prolonged, remaining elevated for 30 min. Activation of Erk2 appeared to be necessary for IL-2 stimulation of proliferation, as deletion of a region of the cytoplasmic domain of the IL-2 receptor β chain, essential for IL-2 stimulation of proliferation, abolished Erk2 activation by IL-2. Furthermore, cells that had been deprived of cytokine for 24 h were then refractory to IL-2 stimulation of both Erk2 activity and proliferation. However, elevation of Erk2 activity was not sufficient to stimulate proliferation, as protein kinase C activation stimulated Erk2 activity but not DNA synthesis. Also, cells exposed to IL-2 in the presence of rapamycin showed full Erk2 activation but not DNA synthesis. These data suggest that IL-2 must stimulate both Erk2 activity and a further pathway(s) to trigger cell proliferation.

IL-2 is a polypeptide growth factor secreted by antigen-activated helper T lymphocytes, which stimulates proliferation and effector functions of a variety of cells of the immune system (1). IL-2 interacts with cell surface receptors, composed of at least three cell surface proteins, to stimulate cells. High affinity receptors are formed from p55 α chains, p75 β chains, and p64 γ chains, whereas intermediate affinity receptors consist of β and γ chains (2). IL-2 occupancy of either high (3), or intermediate (4) affinity receptors can stimulate cell proliferation. Two regions of the cytoplasmic domain of the IL-2 receptor β chain have been shown by mutational analysis to be important for stimulation of proliferation. Deletion of a serine-rich region (amino acids 267–322) results in a receptor that fails to stimulate immediate early gene expression (5) or proliferation (6) when expressed in an IL-3–dependent cell. An acidic domain (amino acids 313–382) is required for proliferation of such cells at an optimal rate in response to IL-2, and for optimal IL-2 stimulation of immediate early gene expression (5, 7).

Several rapid signals that follow IL-2 receptor triggering have been identified. Tyrosine phosphorylation of a number of substrates, including the serine/threonine kinase Raf-1 (8), has been described. The acidic domain of the β chain of the receptor can physically associate with the lymphoid-specific protein tyrosine kinase Lck (9) and IL-2 stimulates the activity of Lck (9). IL-2 has also been shown to increase the level of activated, GTP-bound Ras protein in several cell types, including normal lymphocytes (10), T cell lines (11), and IL-3–dependent cells expressing IL-2 receptors (12, 13). Both the serine-rich and the acidic domains of the IL-2 receptor

β chain are required for this stimulation of Ras GTP accumulation (13). Similar Ras activation has been observed after cell stimulation by IL-3, IL-5, and GM-CSF all of which, like IL-2, interact with receptors of the hemopoietin family (14).

In nonlymphoid cells, activated Ras has been shown to stimulate the phosphorylation and activation of members of the mitogen-activated protein (MAP) kinase family of serine/threonine protein kinases (15). These kinases are activated by multiple signaling pathways, including tyrosine kinase receptors, receptors coupled to heterotrimeric G proteins, and activators of protein kinase C (16). MAP kinases have been shown to phosphorylate, and activate the transcription factors c-jun (17), p62[TCF] (18), and myc (19), which regulate immediate early gene transcription and cell cycle entry. Their activation requires phosphorylation on both threonine and tyrosine (20), which can be catalyzed by a single enzyme MAP kinase kinase (MAPKK) (21). MAPKK itself is thought to be activated by Raf (22). Despite stimulation of Ras activation, the expression level of Raf (23) and Raf phosphorylation (8, 23), by IL-2, MAP kinase phosphorylation in response to IL-2 was not observed in either IL-3–dependent cells expressing IL-2 receptors (24), or in an IL-2–dependent cell line (25). IL-3, IL-5, and GM-CSF have been shown to stimulate MAP kinase phosphorylation and activation (26). Here we report that IL-2 does stimulate rapid, sustained phosphorylation and activation of Erk2, the 42-kD member of the MAP kinase family, in IL-3–dependent cells expressing either high, or intermediate affinity IL-2 receptors. Erk2 stimulation is necessary but not sufficient for stimulation of proliferation of these cells.

Materials and Methods

Recombinant human IL-2 was purchased from Eurocetus (Harejield, UK) and PMA from Sigma Immunochemicals (St. Louis, MO). Conditioned medium from transfected plasmacytoma cells (27) was used as a source of IL-3. 2RB anti–human IL-2 receptor β chain antibody was a gift from Dr. M. Kamio (Kyoto University, Kyoto, Japan), TAC anti–human IL-2 receptor α chain antibody was a gift from Professor T. Waldmann (National Institutes of Health, Bethesda, MD), and rapamycin was a gift from Dr. B. Weston (Smith Kline Beecham, London, UK).

Cell Lines.   BAF3 cells (28) and their derivatives were cultured in DMEM with 10% fetal bovine serum and 0.1% IL-3 conditioned medium, at a density below 4×10^5 cells/ml in a 10% CO_2 humidified incubator. BOE9 and W4E9 cells were constructed as previously described (29). P75 S mutant cells were constructed as follows: a BamHI fragment of the IL-2 receptor β chain cDNA (gift of Professor T. Taniguchi, Osaka University, Osaka, Japan) was inserted in pUC18/13 (30). p75 pUC18/13 was digested with SacI and AflII, filled in with T4 DNA polymerase and religated. The resulting BamHI fragment was ligated into the retroviral vector pBABE Puro (31) which was transfected into Ψ2 packaging cells (32). Supernatant from these cells was used to infect BAF3 cells, which were then selected in 4 μg/ml puromycin (Sigma). Bcl2-15-p75-1 cells were constructed by infecting Bcl2-15 (33) cells with an identical recombinant retrovirus encoding wild-type p75. Proliferation and cell cycle analyses were performed as previously described (33).

Erk2 Measurement.   Samples were prepared by washing 4×10^6 cells per determination twice in DMEM/10% fetal bovine serum, then resuspending them in 50 μl and pretreating as described. A further 50 μl of the same medium containing cytokine was then added and the cells were incubated for the time indicated at 37°C, in a 10% CO_2 humidified incubator. 1 ml of ice-cold PBS was then added and the cells were pelleted by centrifugation for 30 s at 6,500 rpm in a microfuge. Cell extracts were prepared as previously described (15). Western blots and immunoprecipitations using the Erk2-specific antiserum 122 were performed as previously described (15, 34) except that 50 μg of protein was immunoprecipitated and the protease inhibitors used were 1 μg/ml antipain, 1 μg/ml elastinal, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin A, and 0.5 mM PMSF.

Results and Discussion

Stimulation of Erk2 Kinase Activity by IL-2.   The establishment of derivatives of the murine IL-3–dependent cell line BAF3 which express either high or intermediate affinity, IL-2 receptors, by expression of human β chain, or α and β chains, of the IL-2 receptor, has been described previously (29). The cell line expressing intermediate affinity receptors (BOE9) proliferated half maximally in response to 50 pM IL-2, whereas W4E9, which expresses high affinity receptors, responded half maximally to 3 pM IL-2 (29). These IL-3–dependent cells therefore respond at lower levels of IL-2 receptor occupancy than activated primary T cells. Fig. 1 *A* shows that in both BOE9 and W4E9 cells, IL-2 could stimulate Erk2 phosphorylation, as monitored by reduced mobility on an SDS polyacrylamide gel (15). This increase in Erk2 phosphorylation was paralleled by an increase in Erk2 kinase activity (Fig. 1 *B*). Stimulation was rapid, being maximal within 5 min, and sustained being detectable after 20 min in BOE9
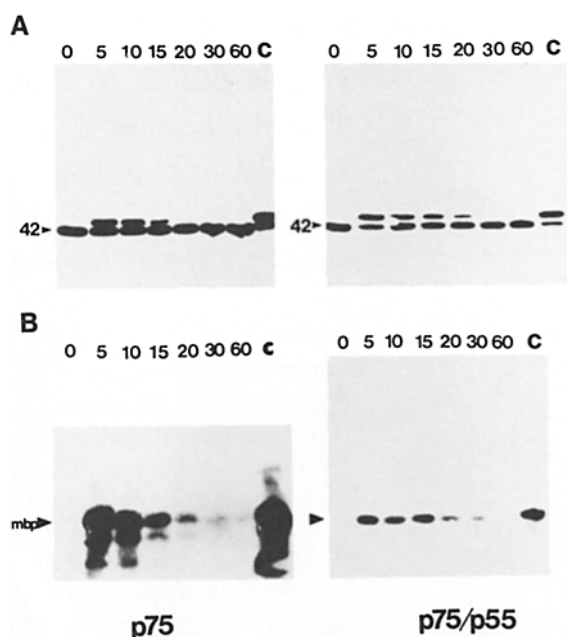
Figure 1.   Time course of Erk2 stimulation by IL-2. BOE9 cells, expressing the p75 β chain of the IL-2 receptor (*p75*), and W4E9 cells, expressing the p75 β chain and p55 α chains (*p75/p55*), were exposed to 250 nM IL-2 for the time indicated (min) or 5% IL-3 conditioned medium (*c*) for 10 min. Erk2 gel mobility (*A*) and kinase activity were then determined. (*A*) Position of the 42-kD unphosphorylated Erk2; (*B*) position of the myelin basic protein kinase substrate (*mbp*).

cells and 30 min in W4E9 cells (Fig. 1, *A* and *B*). Sustained MAP kinase activation has been correlated with the ability of factors to stimulate DNA synthesis (35). As previously reported (26), IL-3 also stimulated MAP kinase phosphorylation and activity (Fig. 1, *A* and *B*). Quantitation of the kinase assay of Fig. 1 *B* showed a 16-fold activation of kinase activity by IL-2, after 5 min, in both BOE9 and W4E9 cells, compared with 20–40-fold activation observed with IL-3. Stimulation of Erk2 phosphorylation by IL-2, but not by IL-3, could be inhibited in W4E9 cells by a combination of anti-α and anti-β chain antibodies (data not shown).

The level of IL-2 required to stimulate Erk2 activity half maximally was somewhat higher than that required to stimulate proliferation of the two cell lines. Fig. 2 shows that 12 nM IL-2 stimulated half-maximal Erk2 activation in BOE9 cells, compared with 50 pM required for half-maximal proliferation. W4E9 cells required 80 pM IL-2 for half-maximal Erk2 stimulation, compared with 3 pM required for proliferation. Thus, the level of occupancy of either intermediate or high affinity IL-2 receptors that led to stimulation of Erk2 activity was higher than that required for stimulation of proliferation.

Erk2 Stimulation Is Necessary for Optimal Stimulation of Proliferation by IL-2.   To further investigate the role of Erk2 phosphorylation in the stimulation of cell proliferation by IL-2, we constructed a cell line expressing the IL-2 receptor β chain deleted in a serine-rich region of the cytoplasmic tail. Surface expression of the mutant β chain was confirmed by staining with the mAb 2RB (data not shown). Fig. 3 *A* shows that IL-2 was unable to stimulate Erk2 phosphorylation in cells expressing this mutant receptor and, as previously described
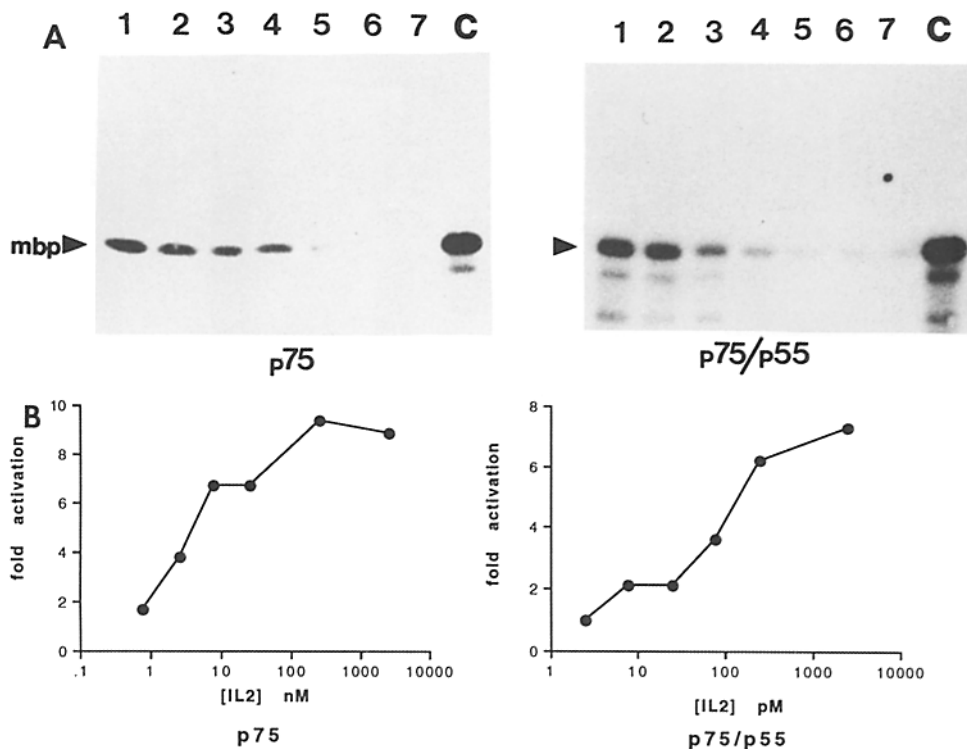
Figure 2. Dose dependence of Erk2 kinase activation by IL-2. (*A*) BOE9 cells (*p75*) were exposed to IL-2 at 2,500 nM (lane *1*), 250 nM (lane *2*), 25 nM (lane *3*), 7.9 nM (lane *4*), 2.5 nM (lane *5*), 790 pM (lane *6*), none (lane *7*), and 5% IL-3 (lane *c*). W4E9 cells (*p75/p55*) were exposed to IL-2 at 2.5 nM (lane *1*), 250 pM (lane *2*), 79 pM (lane *3*), 25 pM (lane *4*), 7.9 pM (lane *5*), 2.5 pM (lane *6*), none (lane *7*), and 5% IL-3 (lane *c*) for 10 min. Erk2 kinase activity was then determined. The position of the myelin basic protein kinase substrate (*mbp*) is indicated. (*B*) Quantitation of the data of *A*.

(6), was also unable to stimulate cell proliferation. The effect of prolonged cytokine removal upon IL-2 ability to stimulate Erk2 phosphorylation was then investigated using a cell line Bcl2-15-p75-1. These cells express both the human Bcl2 oncogene product and the β chain of the IL-2 receptor. Removal of IL-3 or IL-2 from such cells results in a population that remains viable, but arrested in the G1 phase of the cell cycle (33). This allows the cells to be starved of cytokine without complication of experiments by the onset of apoptosis. Removal of cytokine from these cells does not affect the level of expression of the IL-2 receptor β chain (data not shown). Fig. 3 *B* demonstrates that 24 h after IL-3 removal from these cells, IL-2 and IL-3 show a reduced ability to stimulate rapid Erk2 phosphorylation, the IL-2 response being lost earlier. In cells starved of IL-3, IL-3 or IL-2 readdition was unable to stimulate DNA synthesis (Fig. 3 *B*). The extent of downregulation of DNA synthesis stimulation was identical to the decrease in Erk2 stimulation (Fig. 3 *B*). Thus, in both cells expressing a mutant IL-2 receptor and in 24-h starved cells, the loss of rapid IL-2 stimulation of Erk2 was correlated with a loss of IL-2 stimulation of cell proliferation.

Erk2 Stimulation Is Not Sufficient for Stimulation of Proliferation. To examine whether Erk2 stimulation was sufficient to stimulate proliferation of these cells, they were treated with tetradecanoyl phorbol acetate (TPA), an activator of protein kinase C that has been shown to activate MAP kinases in other cells. TPA stimulated Erk2 in W4E9 cells to a similar level as IL-2 and IL-3, as ascertained both by reduced gel mobility (Fig. 4 *A*) and increased kinase activity (data not shown).

However, TPA failed to stimulate proliferation of the same cells (Fig. 4 *A*). The effect of the macrocyclide, immunosuppressive agent rapamycin on IL-2 stimulation of Erk2 activity and proliferation was then monitored. Fig. 4 *B* shows that rapamycin had no effect on the stimulation of Erk2 phosphorylation by IL-2 or IL-3 in W4E9 cells. It also did not inhibit IL-2 or IL-3 stimulation of Erk2 activity (data not shown). However, rapamycin inhibited IL-2 and IL-3 stimulation of DNA synthesis in these cells, without affecting preservation of cell viability (Fig. 4 *B*). These data demonstrate that stimulation of Erk2 activity alone is not sufficient to trigger cell proliferation in the BAF3 cell line. Furthermore, it can be concluded that IL-2 and IL-3 must stimulate an additional, rapamycin-sensitive pathway as well as Erk2, when they stimulate cell proliferation.

The molecular pathway by which triggering of the IL-2 receptor leads to stimulation of Erk2 remains to be determined. Activated Ras has been shown to stimulate Erk2 in fibroblasts, at least partly by a protein kinase C–dependent pathway (15). A similar mechanism might apply to IL-2, which does stimulate Ras.GTP accumulation in IL-3–dependent cells expressing IL-2 receptors (12, 13). However, IL-2 stimulation of cell proliferation can occur in the absence of protein kinase C (36). The fact that cells become refractory to IL-2 stimulation of Erk2 after prolonged cytokine removal, may explain the discrepancy between the stimulation reported here and the previous report that IL-2 was unable to stimulate MAP kinase in similar cells (24). The cytokine-deprived cells retain IL-2 receptor and Erk2 expression, and should there-
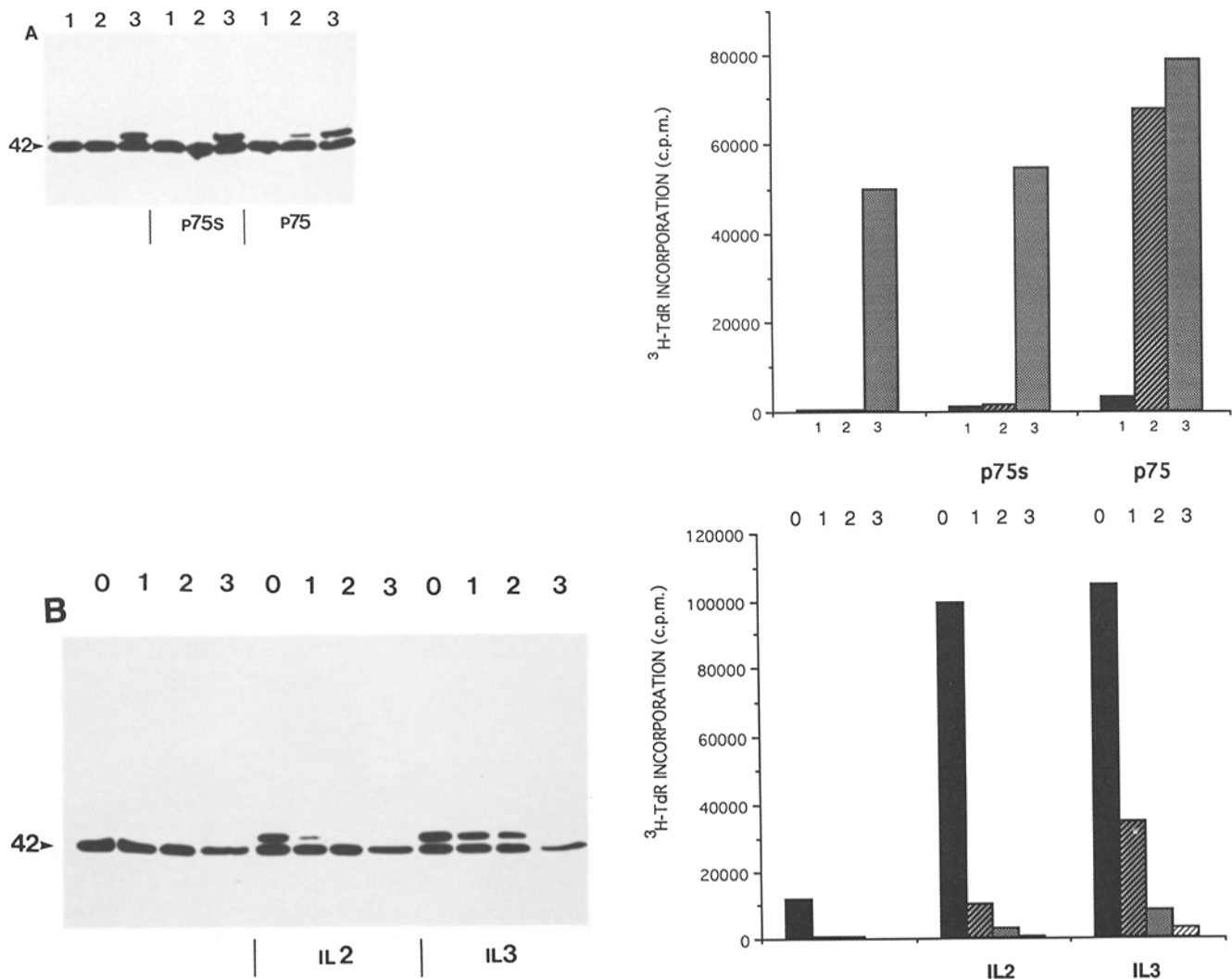
Figure 3. Erk2 stimulation is necessary for stimulation of proliferation. (*A*, *left*) Parental BAF3 cells, cells expressing the IL-2 receptor β chain p75 lacking the serine-rich region (*p75s*) and BOE9 cells (*p75*) were exposed to no factor (lane *1*), 2,500 nM IL-2 (lane *2*), or 5% IL-3 (lane *3*) for 10 min. Erk2 mobility was then measured. (*A*, *right*) Proliferation of the same cells exposed to no factor (lane *1*), 25 nM IL-2 (lane *2*), or 1% IL-3 (lane *3*) for 24 h, then pulsed with [^{3}H]TdR for 4 h. (*B*, *left*) Bcl2-15-p75-1 cells were deprived of IL-3 for 0, 1, 2, or 3 d, as indicated. They were then exposed to no factor, 2,500 nM IL-2 or 5% IL-3, as indicated, for 10 min, and Erk2 mobility was measured. (*B*, *right*) Bcl2-15-p75-1 cells, deprived of IL-3 for 0, 1, 2, or 3 d, as indicated, were exposed to no factor, 250 nM IL-2, or 1% IL-3 in the presence of [^{3}H]TdR for 24 h.

fore be useful tools in the identification of key components on the signaling pathway between receptor and kinase. The fact that rapamycin inhibits IL-2 stimulation of proliferation, without affecting MAP k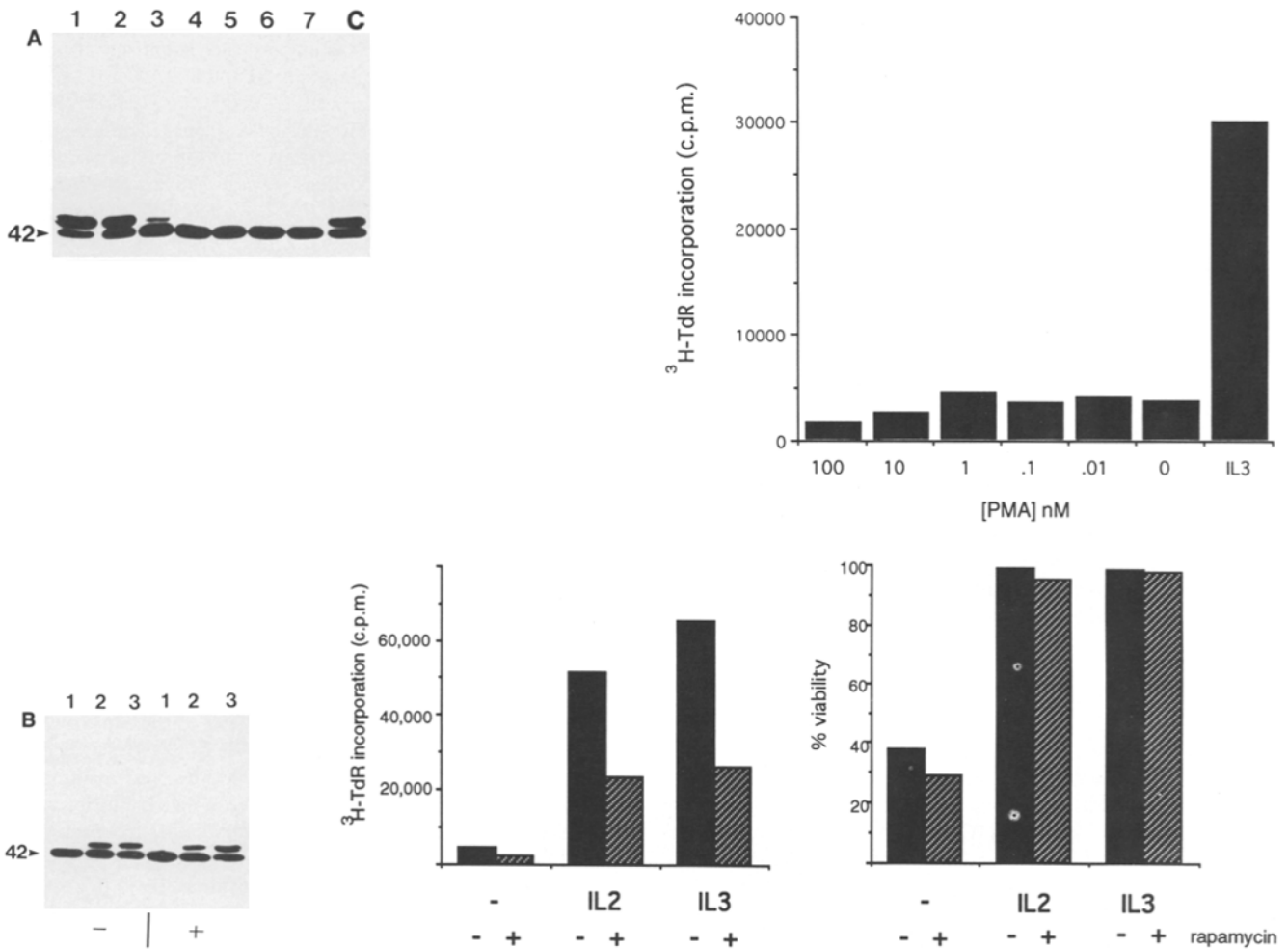inase activation, suggests that a second, rapamycin-sensitive pathway is also required for IL-2 stimulation of proliferation. This probably involves stimulation of the serine/threonine kinase p70 S6 kinase, the stimulation of which by IL-2 (24, 25) is inhibited by rapamycin.

Figure 4. Erk2 stimulation is not sufficient for stimulation of proliferation. (*A, left*) W4E9 cells were exposed to PMA at 100 nM (lane *1*), 10 nM (lane *2*), 1 nM (lane *3*), 100 pM (lane *4*), 10 pM (lane *5*), 1 pM (lane *6*), no factor (lane *7*), or 5% IL-2 (lane *c*) for 10 min. Erk2 mobility was then measured. (*A, right*) W4E9 cells were exposed to the concentration of PMA indicated, or 1% IL-3 for 24 h, then pulsed with [³H]TdR for 4 h. (*B, left*). Control W4E9 cells (−), or cells preincubated with 200 ng/ml rapamycin (+), were exposed to no factor (lane *1*), 25 nM IL-2 (lane *2*), or 5% IL-3 (lane *3*) for 10 min. Erk2 mobility was then determined. (*B, right*). W4E9 cells were incubated with 2.5 nM IL-2 or 1% IL-3 in the absence or presence of 10 ng/ml rapamycin, as indicated, for 24 h. Cell viability and [³H]TdR incorporation after a 4-h pulse were then measured.

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