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Immunology

Expression of v-*src* in a murine T-cell hybridoma results in constitutive T-cell receptor phosphorylation and interleukin 2 production

(oncogene/protein-tyrosine kinase/lymphokine/signal transduction/protein kinase C)

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ABSTRACT **Ligand binding to the T-cell antigen receptor results in phosphatidylinositol hydrolysis and the resultant activation of protein kinase C, as well as the activation of a receptor-coupled protein-tyrosine kinase. As a model for tyrosine kinase activation in T cells, we used retroviral gene transfer to express the v-*src* oncogene in an antigen-specific murine T-cell hybridoma. Clones that expressed v-*src* mRNA demonstrated constitutive tyrosine phosphorylation of several cellular substrates, including the ζ chain of the T-cell receptor, and constitutive interleukin 2 production. Thus, expression of a constitutively active protein-tyrosine kinase such as pp60$^{v\text{-}src}$ appears to be sufficient to induce the expression of at least one gene critical to the process of T-cell activation.**

The biochemical events that occur following perturbation of the T-cell antigen receptor (TCR) include the activation of protein kinase C (PKC) (reviewed in ref. 1) and a nonreceptor protein-tyrosine kinase (PTK) (2, 3). How activation of these two kinases results in later events in lymphocyte activation, such as alteration of gene expression and lymphokine production, is poorly understood. The observation that pharmacologic activation of PKC with phorbol esters, generally in conjunction with a calcium ionophore, leads to lymphokine production has led to the conclusion that phosphatidylinositol breakdown is the receptor-linked biochemical pathway that is physiologically responsible for lymphokine gene expression (1). However, in T-cell hybridomas in which receptor-coupled phosphatidylinositol hydrolysis is impaired, perturbation of the TCR still induces interleukin 2 (IL-2) secretion (4–6). While several explanations can be invoked to explain IL-2 production by these mutant cells, one possibility is that activation of the PTK itself might lead to enhanced gene transcription in the absence of receptor-coupled inositol phosphate generation. A direct role for the PTK pathway in these distal events has not been demonstrated, in part because there are no pharmacologic means to directly activate this enzyme. To help elucidate the potential roles of PTKs in T-cell activation, we sought to develop a model of T-cell activation by expressing a constitutively active PTK.

The v-*src* gene is a mutated and constitutively activated form of the cellular protooncogene c-*src* (7). We used retroviral gene transfer to express v-*src* (8) in the antigen-specific T-cell hybridoma 2B4. We observed that T cells expressing v-*src* constitutively demonstrated two phenotypes typically seen with physiologic T-cell activation: tyrosine phosphorylation of the TCR-ζ chain and production of IL-2.

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METHODS

Production of v-*src*-Expressing T-Hybridoma Cells. Retrovirus-mediated gene transfer was used to express the v-*src* gene in the pigeon cytochrome *c*-specific T-cell hybridoma 2B4. In brief, 10^6 2B4 cells were cocultured with 3×10^6 irradiated packaging cells designated LSNL*src* (generously provided by Robert Overell) in the presence of Polybrene (4 μg/ml) as described (8). In addition, hybridoma cells were cocultured with LSNL, a packaging cell line identical to LSNL*src* except that it lacks the v-*src* gene. After 48 hr, T cells were selected for neomycin resistance with active Geneticin (1 mg/ml; GIBCO) and 72 hr later were cloned by limiting dilution. The clones were then screened both for expression of v-*src* mRNA by slot blot and Northern analysis and surface expression of the TCR by flow cytometry.

Cytoplasmic RNA was prepared as described (9). For Northern analysis, 20 μg of total RNA was electrophoresed in 1% agarose/formaldehyde gels and transferred to nylon membranes (Nytran, Schleicher & Schuell) by capillary action (9). v-*src* mRNA was detected using a 285-base-pair (bp) *Sma* I fragment of the pv*src* plasmid (a gift of Joan Brugge, University of Pennsylvania) that was radiolabeled by using [α-^{32}P] dCTP in a random priming reaction.

Immunoprecipitations. Approximately 10^7 LSNL*src* packaging cells and T cells were washed with methionine-free medium, labeled with [^{35}S]methionine/[^{35}S]cysteine (Tran-^{35}S-label, ICN, 0.1 mCi/ml; 1 mCi = 37 MBq) for 3 hr at 37°C in methionine-free medium with 10% dialyzed fetal bovine serum as described (8). The cells were lysed in buffer containing 50 mM Tris (pH 7.5), 300 mM NaCl, 0.5% Triton X-100, leupeptin at 1 μg/ml, aprotinin at 1 μg/ml, and 2.5 μM *p*-nitrophenylguanidinobenzoate and immunoprecipitated with the anti-pp60$^{v\text{-}src}$ monoclonal antibody (mAb) 327 (kindly provided by Joan Brugge) and rabbit anti-mouse immunoglobulin (Calbiochem). The immunoprecipitates were washed, eluted, and electrophoresed in an SDS/10% polyacrylamide gel.

Enzymatic activity of pp60$^{v\text{-}src}$ was detected by *in vitro* kinase assays using mAb 327 and rabbit anti-mouse immunoglobulin at 4°C for 20 min as described (8, 10, 11). The eluates were electrophoresed in an SDS/7.5% polyacrylamide gel. Anti-phosphotyrosine immunoblotting was performed as described (12–15).

IL-2 Assay. One hundred thousand v-*src*-expressing cells, control cells, or parental hybridoma cells were incubated in medium for 48 hr at 37°C, after which aliquots of supernatant

Abbreviations: IL, interleukin; mAb, monoclonal antibody; PKC, protein kinase C; PTK, protein-tyrosine kinase; TCR, T-cell antigen receptor.

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were removed and frozen. Four thousand IL-2-sensitive CTLL-2 cells were cultured overnight with serial dilutions of these supernatants, then incubated with [*methyl*-^{3}H]-thymidine (5 μCi/ml) for 8 hr and collected onto filters with a semiautomated cell harvester, and incorporated radioactivity was measured by liquid scintillation counting. In antibody blocking experiments, concentrated supernatants from v-*src*-expressing cells were added to 8 × 10^4 IL-2- and IL-4-responsive CT4.R cells (ref. 16; the kind gift of Paul Bohjanen, National Cancer Institute) with or without purified anti-IL-2 receptor mAbs 7D4 and 2E4 (17) or the anti-IL-4 mAb 11B11 (18). In addition, CT4.R cells were cultured with recombinant IL-2 (2 units/ml) or affinity-purified IL-4 (200 units/ml) (kindly provided by W. E. Paul, National Institute of Allergy and Infectious Diseases) in the presence and absence of the above antibodies. IL-2 mRNA was detected using a 0.4-kilobase (kb) *Acc* I–*Hin*dIII fragment of the IL-2 cDNA from pCD-IL-2 (19). Sodium butyrate was prepared as a 1 M stock from butyric acid and titrated to pH 7 with NaOH and was added as indicated (20).

RESULTS

Expression of v-*src* in a T-Cell Hybridoma. Twenty-three T-cell hybridoma clones that were resistant to Geneticin were generated following coculture of these cells with the v-*src* packaging cell line LSNL*src*. These clones were screened for v-*src* mRNA by RNA slot blot analysis and 16 were found to express v-*src* message; seven were found to have no detectable v-*src* mRNA in total cytoplasmic RNA. Two such non-v-*src*-expressing clones (3E4 and 12C3) were retained for further analysis. RNA from all clones hybridized with a probe for the neomycin-resistance gene. Since the surface expression of the TCR is labile in the 2B4 T-cell hybridoma (4–6), the clones generated were also screened by flow cytometry for TCR expression with a clonotypic anti-receptor mAb (14). Clones expressing roughly normal levels of surface TCR included 4A3, 5B2, 7A3, and 9E3. For simplicity, much of the data presented in this paper show results obtained using 4A3 as a representative of this phenotype. However, in all cases the other clones were examined as well to exclude the possibility that 4A3 was exceptional. In contrast to 4A3, clone 4B4 expressed v-*src* but had reduced levels of surface expressed TCR. The mean channel of fluorescence of 4B4 for TCR was ≈20% of that of 2B4 as determined by flow cytometry. This clone also was analyzed further because of this characteristic. Clone 3E4, while not expressing detectable v-*src* mRNA, did express the TCR at levels roughly equivalent to the parental cell line 2B4. The control cells, 2B4LSNL, exhibited similar TCR levels.

As shown in Fig. 1*A*, one species (≈6 kB) of v-*src* mRNA was detectable in v-*src*-expressing clones (8). No hybridization was seen to RNA from the 2B4LSNL control cells or the parental cells (data not shown), reflecting not only undetectable levels of v-*src* mRNA but undetectable levels of c-*src* mRNA in total cytoplasmic RNA derived from these cells. To confirm that a bona fide protein was transcribed in the v-*src*-expressing cells, anti-pp60$^{v\text{-}src}$ mAb 327 was used to immunoprecipitate metabolically labeled pp60$^{v\text{-}src}$ (arrow, Fig. 1*B*) from T-cell clones expressing v-*src* mRNA (e.g., 4A3) and from the packaging cell line, LSNL*src*, used to generate v-*src*-expressing T cells. To determine whether the protein produced in the cells retained its enzymatic activity, *in vitro* kinase activity of immunoprecipitated pp60$^{v\text{-}src}$ was assayed. Kinase activity was readily observed in clones expressing v-*src* message (Fig. 1*C*), as assessed by autophosphorylation of pp60$^{v\text{-}src}$ and phosphorylation of coprecipitated proteins, but not in control cells.

As a further measure of tyrosine kinase activity, anti-phosphotyrosine immunoblot analysis of whole cell lysates was performed. The extent of phosphorylation of endogenous substrates was far greater in v-*src*-expressing clones than in parental 2B4 cells, 2B4LSNL (Fig. 2*A*), or 3E4 (data not shown). Previous studies have demonstrated that the 21-kDa tyrosine-phosphorylated protein detected in whole cell lysates of activated, but not resting, T cells is the TCR-ζ chain (2, 3, 13, 15). The observation that 4B4 cells, which had reduced surface expression of TCR, also had reduced levels of phosphorylation of this substrate raised the suspicion that the 21-kDa substrate evident in v-*src*-expressing hybridomas was TCR-ζ. To test this possibility, we isolated the TCR from these v-*src*-expressing cells by using an anti-TCR-α-chain mAb, A2B4-2, and immunoblotted with anti-phosphotyrosine antibodies. In unstimulated, parental 2B4 cells and

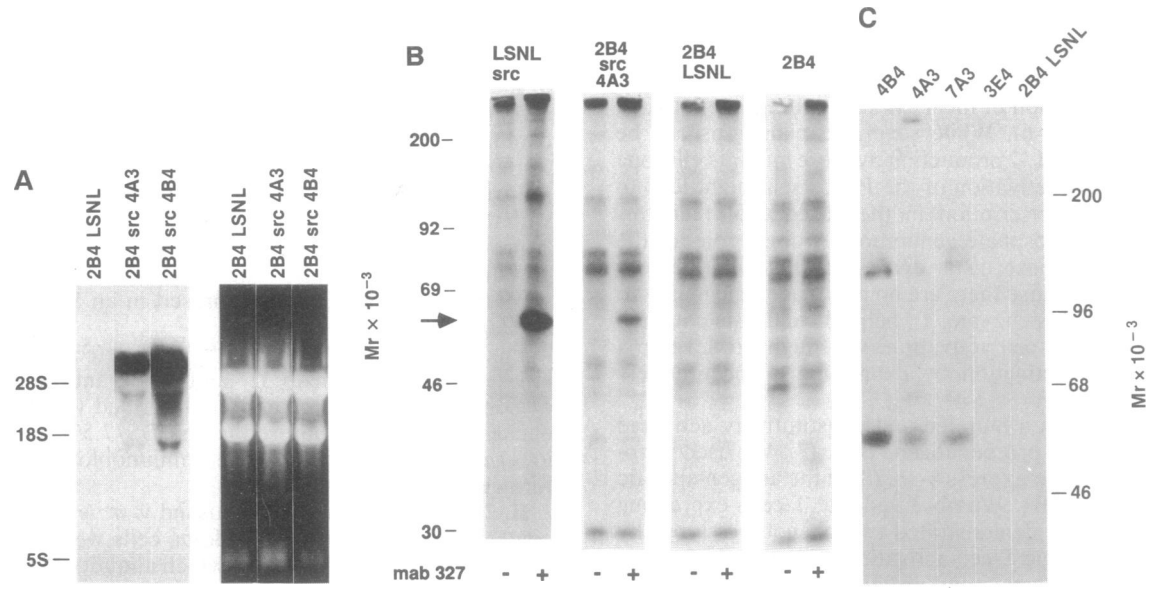

FIG. 1. Expression of pp60$^{v\text{-}src}$ in T cells. (*A*) Northern analysis. Ethidium bromide staining of the gel is shown on the right. Positions of 28S, 18S, and 5S rRNA are included as size markers. (*B*) Immunoprecipitation of metabolically labeled pp60$^{v\text{-}src}$. The lanes corresponding to the packaging cell line represent autoradiograms exposed for 1 day, while the lanes corresponding to the v-*src*-expressing T cells were from 3-day exposures. (*C*) *In vitro* kinase assay of immunoprecipitated pp60$^{v\text{-}src}$ from v-*src*-expressing clones and control cells.

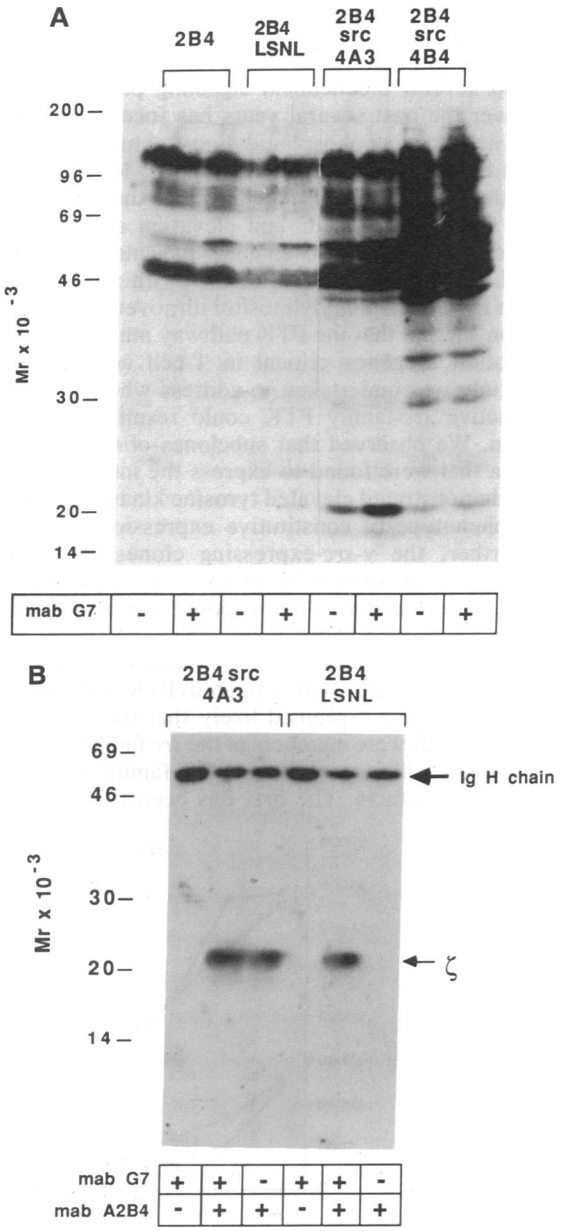

FIG. 2. Tyrosine-phosphorylated substrates in v-*src*-expressing T cells. (*A*) Anti-phosphotyrosine immunoblotting of whole cell lysates. (*B*) Anti-phosphotyrosine immunoblotting of immunoprecipitated TCR. Cells were stimulated or not with the anti-Thy-1 mAb G7. H, heavy.

2B4LSNL cells (Fig. 2*B*), phosphorylation of TCR-ζ was barely detectable. Following activation with a stimulatory mAb, G7, which binds the Thy-1 molecule, tyrosine phosphorylation of this subunit of the TCR was greatly enhanced (Fig. 2*B*). However, in 4A3 and in all other v-*src*-expressing clones that also expressed surface TCR, TCR-ζ was constitutively phosphorylated. In the experiment shown here, activation of 4A3 cells with mAb G7 resulted in little enhancement of constitutive phosphorylation of the TCR-ζ. However, in most experiments with 4A3 and other clones (as well as in Fig. 2), some increase in TCR-ζ phosphorylation was observed following stimulation with G7.

Spontaneous IL-2 Production in v-*src*-Expressing T Cells. Since TCR-ζ phosphorylation is a biochemical marker of physiologic T-cell activation, we speculated that v-*src*-expressing T cells might exhibit other attributes of activated T cells, such as production of lymphokines. A bioassay using IL-2-sensitive CTLL-2 cells showed that in the absence of an exogenous activating agent, parental 2B4 and control 2B4LSNL cells failed to secrete detectable levels of lymphokine (Fig. 3*A*). In contrast, spontaneous growth factor production was noted in seven independent clones that expressed v-*src*, whereas those clones that expressed no v-*src* mRNA (3E4 and 12C3) produced none spontaneously. To ascertain that the growth factor produced by these cells was indeed IL-2, mAb blocking studies were performed. Concentrated supernatant from 4A3 cells was added to IL-2- and IL-4-responsive CT4.R cells in the presence or absence of purified anti-IL-2 receptor mAbs or the anti-IL-4 mAb. To confirm the specificity and efficacy of these antibodies, recombinant IL-2 or purified IL-4 was added to CT4.R cells in the absence or presence of either anti-IL-4 or anti-IL-2 receptor antibodies. We concluded that the growth factor was IL-2, since anti-IL-2 receptor antibodies, but not anti-IL-4 antibodies, inhibited its effect in the bioassay (Fig. 3*B*).

Because relatively low levels of IL-2 were spontaneously secreted by v-*src*-expressing cells (compared to levels produced by T cells that had been stimulated with mAb G7; Fig. 4), we sought to amplify the constitutive levels of secretion

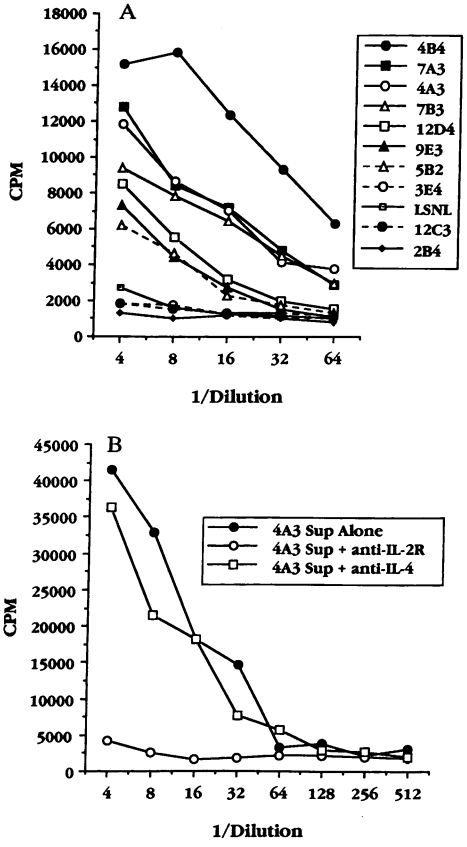

FIG. 3. Constitutive IL-2 secretion in v-*src*-expressing T cells. (*A*) Spontaneous growth factor production by different v-*src*-expressing clones. Culture supernatants from v-*src*-expressing and various control cells were harvested and assayed for IL-2 growth factor activity. (*B*) The growth factor produced by the v-*src* transfectants is IL-2. Supernatant (Sup) from 4A3 cells was collected after 24 hr of growth in medium alone and concentrated 10-fold with a Centricon 10 microconcentrator (Amicon). Serial dilutions of this concentrated supernatant were added to CT4.R cells in the presence of medium alone, anti-IL-2 receptor (anti-IL-2R) mAbs, or an anti-IL-4 mAb, as indicated. Incorporation of [^{3}H]thymidine by the cells was then measured. In the same experiment (data not shown), recombinant IL-2 (2 units/ml) or affinity-purified IL-4 (200 units/ml) was added to CT4.R cells in the absence or presence of either anti-IL-4 or anti-IL-2 receptor antibodies, to confirm the specificity and efficacy of these antibodies.

by incubating cells with sodium butyrate. This agent has been shown to enhance the expression of transfected genes in both stable and transient expression systems (20). When v-*src* expressing T-cell hybridomas were incubated overnight with sodium butyrate, the level of v-*src* mRNA doubled (1.98 ± 0.5; $P < 0.01$, $n = 4$; data not shown). No effect of butyrate was seen on the hybridization of the v-*src* probe to RNA from control cells. Concomitant with an increase in v-*src* mRNA, sodium butyrate also induced an ≈3-fold increase in IL-2 mRNA in v-*src*-expressing cells (data not shown). In contrast, butyrate failed to induced IL-2 mRNA in 2B4LSNL, indicating that the effect of butyrate was not to nonspecifically upregulate IL-2 message levels. When v-*src*-expressing T cells were incubated with sodium butyrate, the constitutive level of IL-2 production as measured in the bioassay was increased 4- to 8-fold (Fig. 4), whereas butyrate had no effect on parental 2B4 cells or 2B4LSNL. In addition, sodium butyrate did not increase the amount of IL-2 that non-v-*src*-expressing cells produced when simultaneously stimulated with mAb G7 (data not shown). In some of the T-cell clones expressing relatively normal levels of surface TCR (4A3 and 7A3), the amount of IL-2 that was produced in the presence of sodium butyrate was similar to or even greater than that produced in response to optimal concentrations of mAb G7. Simultaneous stimulation of butyrate-treated v-*src*-expressing cells with mAb G7 caused no further increase in secretion of IL-2 (data not shown). As expected, due to the reduced levels of surface TCR, mAb G7 had little effect on IL-2 secretion in clone 4B4 (21).

DISCUSSION

As with many receptors, the TCR is directly or indirectly coupled to several biochemical signaling pathways. Most interest over the past several years has focused on two of these; the activation of phosphatidylinositol turnover and the activation of one or more tyrosine kinases. Much evidence suggests that following TCR engagement, the former pathway, via activation of PKC and elevation of intracellular calcium levels, can lead to changes in gene transcription (reviewed in ref. 1). However, studies with mutant T cells that fail to show phosphatidylinositol turnover after receptor stimulation suggest that the PTK pathway may also result in the activation of genes critical in T-cell activation. The present study was undertaken to address whether a constitutively active *src*-family PTK could result in IL-2 gene expression. We observed that subclones of the 2B4 T-cell hybridoma that were found to express the introduced v-*src* gene and demonstrated elevated tyrosine kinase activity also had the phenotype of constitutive expression of the IL-2 gene. Further, the v-*src*-expressing clones uniquely responded to graded doses of butyrate by the acute induction of IL-2 gene expression. This response correlated with an increase in expression of the v-*src* gene.

Are the phosphorylation events initiated by v-*src* expression analogous to the TCR-activated PTK pathway? We chose v-*src* because it seemed likely that the PTK(s) activated via the TCR were members of the *src* family of kinases. 2B4 cells express three members of this family: the *lck*, *yes*, and *fyn* gene products. The first has been thought of as a

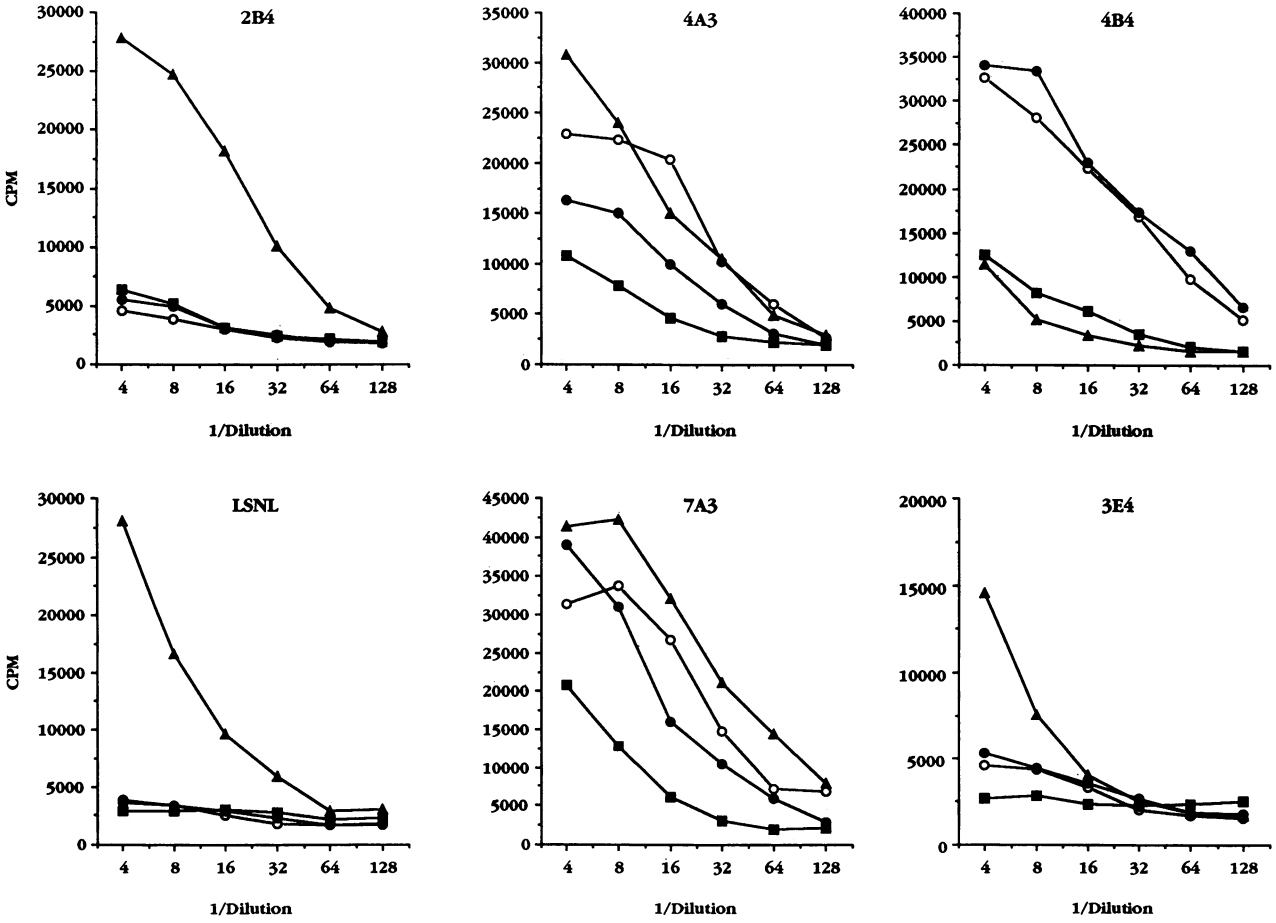

FIG. 4. Sodium butyrate increases spontaneous IL-2 production by v-*src*-expressing hybridomas. Sodium butyrate was added at 0 μM (●), 50 μm (□), or 100 μM (○) and the cells were incubated for 2 days. Doses of butyrate higher than 100 μM were not used, because of toxicity in the bioassay. Aliquots of cells were also stimulated with an optimal concentration of mAb G7 (1:50 dilution of ascites, ▲) with no sodium butyrate. The supernatants were assayed for IL-2.

candidate in T-cell activation because of its tight noncovalent association with CD4 and CD8 (22, 23). Crosslinking of CD4 molecules activates pp56^{lck} and results in the tyrosine phosphorylation of TCR-ζ (24, 25). However, crosslinking of CD3 does not activate pp56^{lck}, nor is pp56^{lck} coimmunoprecipitated with the TCR. Recently, *fyn* kinase has emerged as the most likely candidate in this pathway with the demonstration that it is specifically associated with the TCR (26). The extent that the v-*src* model described here provides an accurate reflection of T-cell activity events will ultimately depend upon how closely the v-*src*-encoded kinase mimics the substrate spectrum of the TCR-activated PTK(s). Several of the substrates constitutively phosphorylated in the v-*src*-expressing T cells are identical to those seen after receptor stimulation, including the TCR-ζ chain. Also, IL-2 production in these v-*src*-expressing cells was blocked by cyclosporin A at doses that inhibit TCR-mediated IL-2 production (J.J.O'S. and J.D.A., unpublished data). Our data are consistent with the notion that an activated PTK is capable of inducing IL-2 production, and at least several of the characteristics of TCR-activated pathways are reproduced in this model. However, does expression of v-*src* mimic completely the response of the T cells to antigen either biochemically or functionally? The answer is clearly no for several reasons. First, the amount of IL-2 made by v-*src*-expressing cells was small. This level was enhanced by butyrate treatment and achieved the level induced by the anti-Thy-1 mAb G7. The level of IL-2, however, was still less than that produced when antigen was used as a stimulus (27). Moreover, stimulation of v-*src*-expressing T cells with antigen, anti-CD3ε mAb, or G7 resulted in enhanced IL-2 secretion (Fig. 4 and unpublished observations), suggesting that the effect of pp60$^{v\text{-}src}$ is suboptimal. Second, while the global tyrosine phosphorylation is high in v-*src*-expressing cells, the finding that tyrosine phosphorylation could be enhanced by perturbation of the TCR suggests that the TCR-coupled PTK can complement the function of pp60$^{v\text{-}src}$. Further, despite the high levels of tyrosine phosphorylation in v-*src*-expressing cells, pp60$^{v\text{-}src}$ may be inefficient in phosphorylating certain key substrates. In this regard, we have recently found that certain tyrosine-phosphorated substrates are poorly phosphorylated in v-*src*-expressing cells compared with cells stimulated by perturbation of the TCR (I. Kennedy, J.J.O'S., unpublished data). Taken together these data suggest that pp60$^{v\text{-}src}$ may be suboptimal as a PTK, relative to the physiologically coupled PTK(s). Since multiple biochemical pathways are activated upon perturbation of the TCR, it is clear that expression of v-*src* is an imperfect model for explaining all of T-cell activation. Nonetheless, we find it striking, even given the limitations of such a model, that expression of an activated PTK is sufficient to induce the expression of such a key gene as the IL-2 gene.

Overell *et al.* (8) have reported the expression of v-*src* in lymphocytes after retrovirus-mediated gene transfer. In that study, v-*src*-infected CTLL cells did not proliferate independently of exogenous IL-2, suggesting that the cells did not make the lymphokine. A likely explanation for the differences between that study and ours is that the CTLL cells did not express PTK activity greater than the parental nontransfected cells. In contrast, the v-*src*-expressing 2B4 cells that made IL-2 had a great deal of PTK activity measured both by *in vitro* kinase assay and by immunoblot analysis of tyrosine-phosphorylated substrates.

Our preliminary evidence suggests that PKC is not necessary for IL-2 production in v-*src*-expressing cells, since cells depleted of PKC by prolonged exposure to phorbol 12-myristate 13-acetate had comparable levels of IL-2 mRNA (data not shown). Thus, we feel that the observations made in v-*src*-expressing 2B4 cells support the notion that activation of a *src*-like PTK alone can lead to some of the events that follow TCR stimulation. Nonetheless, it is possible that in physiologic TCR-mediated signaling both the PTK and phosphatidylinositol–PKC pathways lead to changes in gene expression in T cells. Indeed, in dissecting the genetic elements responsible for the regulation of IL-2 gene transcription, separate phorbol-ester and antigen-receptor response elements have been described (27). While evidence is accumulating that suggests PTKs regulate phosphatidylinositol metabolism (28, 29), precisely how activation of these two pathways results in gene expression remains to be elucidated.

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