Inhibition of p70/p85 S6 Kinase Activities in T Cells by Dexamethasone

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Glucocorticoids (GC) are potent immunosuppressive agents that interfere with interleukin-2 (IL-2)-dependent proliferation and IL-2 receptor signal transduction in T lymphocytes through complex mechanisms. Here we report that the basal activity, and IL-2- and phorbol ester-dependent activation of the p70/p85 S6 kinases (referred to collectively as pp70S6k) are inhibited by the glucocorticoid dexamethasone (Dex) in CTLL-20 cells. This Dex-induced inhibition is time- and dose-dependent, appears to be the consequence of pp70S6k dephosphorylation, and requires ongoing transcription. Attempts to establish a link between Dex action and those of known pp70S6k-regulating agents such as phosphatidylinositol 3-kinase, protein kinase A-stimulating agents, calyculin A-inhibited protein phosphatases, and rapamycin have been negative. Additional results with NIH3T3 cells suggest the existence of a T cell-specific blockade of p70S6k by Dex. Implications are 2-fold: 1) pp70S6k inactivation may account for at least part of the immunosuppressive effects of GC in vivo, and 2) GC inactivation of pp70S6k is exerted through a novel, distinct mechanism that does not appear to be linked to any other known pp70S6k regulatory processes. (Molecular Endocrinology 10: 1107-1115, 1996)

INTRODUCTION

After T lymphocyte activation through the T cell antigen receptor, representing a G0 to G1 transition, binding of the cytokine interleukin-2 (IL-2) to its high affinity receptor (IL-2R) is a necessary step for further progression into S phase and, therefore, for T cell proliferation and clonal expansion (1, 2). The IL-2R is a member of the cytokine receptor superfamily, which, unlike other classes of growth factor receptors, lacks intrinsic enzymatic activity. It is accepted that IL-2R signal transduction is initiated by its functional interaction with and rapid activation of nonreceptor protein kinases, which in turn can induce the second messenger- and phosphorylation-mediated activation of various signal-transducing proteins. The IL-2 mitogenic signal is antagonized by inhibitors of tyrosine phosphorylation (3), is independent of the conventional isoforms of protein kinase C (cPKCs) (4–10), and correlates with the activation of c-Ras (4–6, 11), Jak1 and Jak3 (12, 13), and phosphatidylinositol 3-kinase (PI3K) (14–17).

A functional link has been demonstrated between agonist-dependent activation of PI3K and a serine/threonine protein phosphorylation cascade involving the physiological S6 kinases p70/p85S6k (αII/αI forms, collectively referred to as pp70S6k) (18–22) in IL-2-responsive lymphoid cells as well as in other cell systems. PI3K is the only mediator of receptor signaling to pp70S6k yet identified in the IL-2R system (23, 24).

The only two substrates of pp70S6k identified so far are the ribosomal protein S6 (25, 26) and the nuclear factor CAMP response element modulator (27). Serine phosphorylation of the ribosomal protein S6 after pp70S6k activation is triggered by a variety of oncogenic and mitogenic stimuli, and it is implicated in the regulation of protein synthesis (28, 29). Studies using the immunosuppressant rapamycin to specifically block activation of pp70S6k and phosphorylation of S6 and CAMP response element modulator strongly suggest that this kinase has a central role in growth factor-modulated G1 to S cell cycle transition in fibroblasts, hepatoma cells, and T cells (20, 30–35; see Ref. 36 for a review). Interestingly, the severity of the cell cycle arrest in G1/S after pp70S6k inactivation is most notable in lymphoid cells (20, 32).

Activation of pp70S6k correlates with its phosphorylation on serine/threonine residues (37). While numerous efforts directed at the identification of the upstream protein kinase and/or phosphatase activities responsible for the regulation of pp70S6k phosphorylation state in vivo have been unsuccessful, independent studies (30, 32, 38–41) have indicated that the regulation of pp70S6k is independent of the extensively characterized pathway including c-Ras, c-Raf, mitogen-activated, extracellular signal-regulated kinase kinase, mitogen-activated protein kinase, and pp90RSK (reviewed in Refs. 42–44).
Natural and synthetic glucocorticoids (GCs) mediate a wide range of cell and tissue-specific responses. This is usually the result of altered regulation of gene expression at multiple levels, including transcriptional control and changes in mRNA stability. The best known mechanism of action is that mediated through the interaction of GCs with specific soluble glucocorticoid receptors (GRs) in the cytoplasm of target cells (see Refs. 45-49 for reviews). The GR is almost ubiquitously distributed and is a member of a superfamily of hormone-activated nuclear receptors that function as transcriptional modulators. The specificity of response is determined by local prereceptor, receptor, and genomic differences (47, 50).

GCs are used clinically because of their growth-inhibitory actions and most of all because of their potent immunosuppressive (local and systemic) and antiinflammatory properties, which have made them relevant in the treatment of autoimmune and allergic diseases, as well as in prevention of allograft rejection. Unlike other immunosuppressants such as rapamycin, cyclosporin A, or FK506, which each act at a single step of the T cell activation/proliferation process (51-54), immunosuppression by GCs is quite complex, taking place at multiple levels and affecting different cell types in the immune system (reviewed in Ref. 55).

Considering the complexity of the known immunosuppressive effects of GCs, and given that 1) in vivo the T cell population contains cells in different activation stages, 2) the IL-2/IL-2R-mediated signaling is indispensable for eliciting a normal immune response, and 3) pp70S6k appears to mediate its proliferative effects, the question thus arises as to whether GC-inhibitory effects on lymphocytes are exclusively the result of interference with T cell antigen receptor-mediated signal transduction (56-61) or are also exerted through antagonism with IL-2-dependent signaling events. It has been described that dexamethasone (Dex, a synthetic GC) inhibits IL-2-dependent signal transduction and proliferation in phorbol ester-activated blasts through a post-IL-2R mechanism, the nature of which remains unknown (62).

Here we report that in IL-2-dependent CTLL-20 cells, Dex reduces basal pp70S6k activity and antagonizes IL-2- and phorbol ester phorbol myristate acetate (PMA)-dependent activation of pp70S6k in a time- and dose-dependent manner. This appears to occur by inducing dephosphorylation of the enzyme and/or preventing its agonist-dependent phosphorylation. GC effects on pp70S6k require active transcription. Dex does not antagonize IL-2-dependent PI3K activation, and further attempts to link its action with previously described pp70S6k-regulating agents or mechanisms were negative, suggesting a novel mechanism for pp70S6k inhibition.

RESULTS

Dex Blocks Basal and IL-2-Induced Activity of pp70S6k in T Cells in a Time- and Dose-Dependent Manner

Incubation of CTLL-20 cells with 1 μM Dex for increasing periods of time ranging from 1-12 h previous to the addition of IL-2 shows an increasing degree of inhibition of pp70S6k activity both in quiescent and IL-2-stimulated cells (Fig. 1). No consistent effect was detected at shorter timepoints. In contrast, Dex treatment of NIH 3T3 cells for up to 6 h failed to induce a comparable inhibition of pp70S6k in either quiescent or serum-stimulated cells (Fig. 2). Additionally, the magnitude of Dex effects on pp70S6k was dependent on the concentration of steroid (Fig. 3). Inhibition of basal and IL-2-stimulated pp70S6k activity was detected with a Dex concentration in the nanomolar range (10^-8 M) and reached a plateau at concentrations above 10^-7 M, the IC50 being approximately 10^-8 M.

Immunoblot analysis indicated that the levels of pp70S6k in samples were comparable, with no significant time- or dose dependent change in pp70S6k protein amount (Fig. 1 and not shown). However, Dex did inhibit pp70S6k phosphorylation since Western blots

![Fig. 1. Time Course of Dex Effects on Quiescent and IL-2-Stimulated pp70S6k Activity in CTLL-20 Cells](https://academic.oup.com/mend/article-abstract/10/9/1107/2713443)

A. Quiescent cells were treated with 1 μM Dex for the indicated amount of time before lysis. IL-2 stimulations were for the final 30 min. Cells were lysed and immune complexes formed, and the activity of pp70S6k was measured using the ribosomal protein S6 as a substrate. B. Western blot analysis of aliquots of these lysates with antibodies to pp70S6k.
Dexamethasone Inhibits pp70S6k Activity in T Cells

TIME OF Dex TREATMENT (hours)

Fig. 2. Dex Effects on Quiescent and Serum-Stimulated NIH 3T3 Fibroblasts

Quiescent cells were treated with 1 µM Dex for the indicated amount of time before lysis. Serum stimulations were for the final 30 min. Pp70S6k activities were measured by immune complex assay.

Dex CONCENTRATION (µM)

Fig. 3. Dose-Response Effects of Dex on Quiescent and IL-2-Stimulated pp70S6k Activity in CTLL-20 Cells

Quiescent cells were treated with various concentrations of Dex for 12 h and stimulated or not with IL-2 for the final 30 min before lysis. Pp70S6k activities were measured by immune complex assay.

from lysate samples clearly showed a correlation between activity loss and a decrease in the amount of highly phosphorylated, slower mobility forms of pp70S6k and concomitant increase of the less phosphorylated, faster mobility forms (Fig. 1) in both αl-p70 and αl-p85 S6 kinase isoforms.

Dex-Dependent Inhibition of pp70S6k Activity Requires Active mRNA Synthesis

To determine whether Dex effects on pp70S6k were dependent on ongoing transcription, quiescent CTLL-20 cells were treated for different times (1.5, 3, or 6 h) with one of the following: vehicle (ethanol), Dex alone, actinomycin D (RNA synthesis inhibitor) alone, or Dex with actinomycin D. For each condition two plates of cells were used; IL-2 was added to one of them for the last 30 min before lysis, while the other received no additional treatment. As shown in Fig. 4, actinomycin D alone had little effect on basal or IL-2-stimulated pp70S6k activity, while Dex, as expected, inhibited both in a time-dependent fashion. Importantly, the presence of actinomycin D prevented Dex-induced inhibition of pp70S6k. We conclude that active RNA synthesis is necessary for Dex to exert its effects on pp70S6k and that Dex is likely to induce or repress the synthesis of at least one protein able to act as an upstream modulator of pp70S6k.

Dex Effects on Modulators of pp70S6k Activity

We next tried to establish a connection between Dex action and some of the known agents or enzymes shown to be modulators of pp70S6k activity in T cells. PI3K is the only IL-2R-proximal upstream physiological regulator of pp70S6k identified to date, and specific inhibition of its activity prevents agonist-dependent, cPKC-independent activation of pp70S6k in different systems (19, 23, 24). Additionally, there is one instance of Dex affecting agonist (insulin)-dependent PI3K stimulation (63). Thus, PI3K constituted a potential target of Dex effects. As shown in Fig. 5, this was not the case. Quiescent CTLL-20 cells treated with 1 µM Dex for 6 h (a time by which effects on pp70S6k are maximal, see Fig. 1) did not show any change in basal PI3K activity and IL-2-stimulated PI3K activity was not affected.

Fig. 4. Effect of RNA Synthesis Inhibition on the Dex-Induced Inhibition of pp70S6k Activity in CTLL-20 Cells

Cells were starved for 16–18 h and treated with or without the following, as indicated: Dex (1 µM, 1.5, 3, or 6 h), actinomycin D (Act D, 5 µg/ml, 1.5, 3, or 6 h), or IL-2 (100 U/ml, 30 min). Cells were lysed and pp70S6k activities were measured by immune complex assay. The horizontal lines in the graph indicate the activity level of control, untreated cells (lower) and the relative pp70S6k activity of cells treated with IL-2 only (upper).
It has been demonstrated that both rapamycin and cAMP-dependent protein kinase (protein kinase A (PKA))-stimulating agents inhibit agonist-dependent pp70S6k activation in T cells. Therefore we examined the possibility that the effects of Dex were mediated by either of these signaling systems. Previous results from our laboratory indicated that increasing intracellular cAMP levels inhibited IL-2-, PI3K-mediated pp70S6k activation (24). This inhibition occurred on at least two levels, one being at PI3K itself and the other(s) downstream of this enzyme. Although Dex did not affect IL-2-dependent PI3K activation (Fig. 5), the possibility remained that another of the components of the signaling pathway downstream of PI3K, also affected by raising cAMP levels, was targeted by Dex. Maximal inhibition of pp70S6k by raising cAMP levels was only obtained when using a combination of forskolin (25 μM) and 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) (see Fig. 6 and Ref. 24). When using forskolin alone (12.5 or 25 μM), partial inhibition was observed. IBMX alone had insignificant effects (not shown). Dex treatment (1 μM) for 3 h did not potentiate the effects of forskolin as seen by the addition of IBMX (Fig. 6). This result is consistent with Dex not modulating PKA activation. We cannot rule out the possibility that a signaling molecule downstream of PKA, which may mediate one of its many regulated processes, is affected by Dex.

Another possibility was that Dex acted upon or through the same target as rapamycin, namely the FK506-binding protein (FKBP). Such a model would be quite plausible, as FKBP's are known to be an integral part of the inactive GR complex (64). This idea was examined in two ways: first, simultaneous treatment with submaximal concentrations of Dex and rapamycin demonstrated an additive effect, the sum of which though was less than observed with maximal concentrations of Dex or rapamycin alone (Fig. 7A). Second, cells incubated with Dex were further treated with FK506 at a concentration that reverts rapamycin's potent inhibition of pp70S6k by competing for FKBP's (20, 24, 32). Dex inhibition of pp70S6k was not affected by such high concentrations of FK506 (Fig. 7B). Thus, there appears to be no obvious cross-talk between Dex and rapamycin, neither at the level of their primary targets nor downstream of them. Additionally, calyculin A, an inhibitor of phosphatases 2A and 1C and activator of pp70S6k (20), was ineffective in overcoming Dex-induced inhibition of pp70S6k (Fig. 8). This indicates that Dex-mediated inhibition of pp70S6k is not the result of inducing the expression of or activating calyculin A-sensitive phosphatases.

At least two signaling processes are known to regulate pp70S6k activity, pathways dependent on and independent of cPKC isoforms. Like other IL-2-dependent cell lines, CTLL 20 cells contain functional cPKCs capable of being activated by phorbol esters, and we recently showed that IL-2 activates pp70S6k in a cPKC-independent manner (24). Yet, pp70S6k is stimulated to similar degrees by the phorbol ester PMA and IL-2 in these cells, and a variety of situations where cross-talk happens between the cPKC and the GC-signaling networks have been reported (65-67). Results in Fig. 9 demonstrate that, as shown before for IL-2, Dex is able to antagonize PMA-induced pp70S6k activation. Since PMA is still able to fully stimulate MAPK/RSK in Dex-treated cells (not shown), it can be inferred that Dex inhibition of the cPKC-dependent activation of pp70S6k does not occur through
A

Fig. 7. Effect of Rapamycin or Excess FK506 on Dex-Mediated Inhibition of pp70S6K in CTLL-20 Cells
A. Quiescent cells were treated or not with Dex (1 μM, 3 h), IL-2 (100 U/ml, 30 min), and rapamycin. Treatments with Dex for 3 h and/or rapamycin at 0.3 ng/ml were done to induce submaximal inhibition of IL-2-induced pp70S6K activity. B. Quiescent cells were treated or not with Dex (1 μM, 6 h), IL-2 (100 U/ml, 60 min), and FK506 (8 μg/ml, 60 min). In all cases, pp70S6K activities were measured by immune complex assay.

B

Fig. 8. Effect of Calyculin A on Dex-Mediated Inhibition of pp70S6K in CTLL-20 Cells
Quiescent cells were treated or not with Dex (1 μM, 6 h), calyculin A (10 nm, 30 min), and IL-2 (100 U/ml, 30 min). Pp70S6K activities were measured by immune complex assay.

DISCUSSION

The immunosuppressive and antiinflammatory actions of GCs are the basis of their widespread clinical use, which includes the treatment of autoimmune and allergic diseases, and prevention of allograft rejection. GC immunosuppression is actually a complex, multifaceted event, some of which affect lymphocyte proliferation and action.

The treatment of IL-2-dependent cells with Dex induces the time- and dose-dependent inhibition of basal and agonist-stimulated pp70S6K activity. This inhibition is accompanied by dephosphorylation of the two isoforms (α and β) of the enzyme. The observation that basal pp70S6K activity is affected in the same way that the agonist-induced is, indicates that Dex effect is taking place at a postreceptor level. This is in agreement with the work of a direct inhibition of PKC. This result is also consistent with the notion that Dex is targeting a regulatory molecule downstream of the point of convergence of the PI3K- and cPKC-mediated signaling pathways to pp70S6K.

According to these combined results, Dex inactivation of pp70S6K is exerted through a novel mechanism that does not appear to be linked to any other known pp70S6K-regulatory process.
Paliogianni et al. (62) which demonstrates that Dex inhibition of IL-2-dependent proliferation and signal transduction does not result from interference with IL-2 binding to IL-2R or from a loss of cell surface expression of IL-2R. Thus, Dex mode of action is analogous to that of other immunosuppressive agents that also inhibit pp70s6k, namely rapamycin (20, 30, 32, 41, 68) and cAMP (24). In contrast to them, Dex action on pp70s6k is neither as rapid nor as potent as the actions of rapamycin and cAMP, which, in a matter of minutes, are able to reduce pp70s6k activity to below quiescent levels. Of note, both rapamycin and cAMP interfere with the IL-2-dependent G1 to S cell cycle transition in lymphocytes (20, 24). Dex has also been proved to inhibit IL-2-dependent proliferation of T cells as well as an IC50 of 5 x 10^-8 (62), consistent with pp70s6k inhibition. Thus, pp70s6k inactivation may account for at least part of the immunosuppressive effects of GCs in vivo.

Dex-induced inhibition of both basal and IL-2-dependent pp70s6k activity is prevented by simultaneous treatment with actinomycin D, which by itself does not interfere with pp70s6k. Therefore, Dex action requires active transcription (and, presumably, active translation), suggesting a mechanism involving the classical GR-mediated change in gene expression.

The time- and dose-dependence of Dex action, the requirement of ongoing transcription, together with the fact that Dex effects do not involve a loss of pp70s6k protein but a dephosphorylation event(s), lead us to speculate that Dex may specifically induce or suppress the synthesis of at least one protein that acts as an upstream regulator of pp70s6k. Furthermore, considering what is known about pp70s6k regulation (69), the possibility exists that this protein is a pp70s6k phosphatase, or a protein kinase or phosphatase of unknown specificity whose activity on one or more of pp70s6k's upstream regulators would result in pp70s6k inactivation. This idea is compatible with growing evidence that, at any given time, pp70s6k phosphorylation state is the result of a dynamic regulation process, the interplay of regulated upstream kinases and highly active phosphatases (20, 24, 32). The consideration that Dex affects basal pp70s6k activity as well as its activation by IL-2 or PMA suggests that at least one of the affected pp70s6k regulator(s) could be required for the kinase's basal phosphorylation/activation.

Interaction with transcription factors is one means by which the steroid hormone pathway is coupled to signals that function through cell-surface receptors to regulate those transcription factors (46). However, not much is known about signaling pathways under the direct transcriptional control of GCs. Dex has been suggested to inhibit the activation of Ca2+/calmodulin kinase during T cell activation through the induction of protein phosphatase activity (61). As far as protein kinases are concerned, only two serine/threonine kinases have been described so far whose expression is transcriptionally regulated by GCs: snk (70), whose induction by serum is suppressed by GCs, and sgk (71), which is up-regulated by both serum and GCs. These are, along with cdc-2 kinase (72, 73), the first instances of rapid transcriptional control of protein kinases, which are usually subject to posttranslational control mechanisms (phosphorylation/dephosphorylation and/or interaction with second messengers) (74, 75). It has been proposed that snk and sgk represent a novel cross-talk between membrane-linked signaling pathways and intracellular steroid hormone control of gene transcription. Our results are compatible with the notion that Dex effects on pp70s6k in IL-2-dependent T cells might be another example of such cross-talk. If so, what is Dex “talking to”?

The cAMP-dependent protein kinase (PKA) is not activated by IL-2 stimulation in T cells (24, 76). Raising the intracellular levels of cAMP, which results in activation of PKA, can block IL-2-dependent T cell proliferation through a PI3K-dependent serine/threonine phosphorylation cascade (24, 77). Our results show no effect of Dex on PI3K activity or any potentiation of PKA activation effects on pp70s6k by Dex when intracellular CAMP levels were raised shortly before IL-2 stimulation. Dex effects also appear to be independent of FKBP and calyculin A-sensitive phosphatases. These results tell us what Dex is not talking to, at least under the conditions applied. Although these avenues remain open to and deserve some additional exploration, the above posed question remains to be answered and becomes even more intriguing.

While the inhibition of pp70s6k activation in response to IL-2 and cPKC by Dex could be accounted for by effects on the enzyme's basal phosphorylation/activation machinery, the complexity of GC actions in cellular homeostasis does not allow us to rule out the possibility of Dex acting at other levels such as the postreceptor signal transduction process. The lack of effects of Dex on IL-2-dependent PI3K activation suggests that Dex does not induce an overall, indiscriminate block of IL-2R signal transduction but may antagonize it at discrete steps downstream of PI3K. Like rapamycin, but unlike the PI3K inhibitor wortmannin, Dex is also able to inhibit cPKC-mediated pp70s6k activation. Therefore, at least one of the Dex targets in the pp70s6k-signaling pathway could be in the same area where rapamycin acts (26, 27, 35), i.e. downstream of cPKC and PI3K.

It is interesting that a treatment of up to 6 h of NIH3T3 fibroblasts with Dex does not significantly inhibit basal or serum-dependent pp70s6k activity, and a 24-h Dex incubation inhibits pp70s6k activity in these cells only to a degree similar to that achieved in T cells after just 3 h (M. Monfar and J. Blenis, unpublished results). These observations are consistent with the notion that the pp70s6k pathway plays a more dominant role in the proliferation of T cells than in fibroblasts (20), as well as with the occurrence of a cell type-specific effect of Dex on pp70s6k.

GCs are clearly implicated in the regulation of the immune system and, like cAMP, represent a physiological way of regulating IL-2-dependent T cell prolif-
eration that involves inhibition of pp70S6K activity. Our results provide evidence that GC inactivation of pp70S6K is exerted through a novel, distinct mechanism that does not appear to be linked to any other known pp70S6K-regulatory process.

MATERIALS AND METHODS

Acknowledgments

We are grateful to Margaret Chou and Timothy Grammer for their suggestions and comments on the manuscript. We also thank M. K. Gately, S. N. Seghal, B. Druker, and T. M. Roberts for providing various reagents.

Received August 15, 1995. Revision received June 6, 1996. Accepted June 21, 1996.

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This work was supported by Public Health Service Grants GM-51405 from the National Institutes of Health and Harvard Medical School Funds for Discovery. J.B. is an Established Investigator of the American Heart Association.

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Cell Culture

The murine IL-2 dependent CTLL-20 cell line was cultured in DMEM supplemented with L-arginine-HCl, l-asparagine-H2O, folic acid, essential vitamins, nonessential amino acids, 10% (vol/vol) heat-inactivated FBS, 10 mM HEPES (pH 7.2), 2 mM glutamine, 50 μM β-mercaptoethanol, and 20 U/ml penicillin/20 μg/ml streptomycin with 10 U/ml recombinant human IL-2 (referred to as IL-2). Cells were deprived of IL-2 for 14–18 h before treatment with drugs and/or stimulation with IL-2 (100 U/ml).

NIH 3T3 cells were cultured in DMEM supplemented with 10% (vol/vol) calf serum and 20 U/ml penicillin/20 μg/ml streptomycin. Cells were starved for 24 h at 0.5% serum before treatment with drugs and/or stimulation with calf serum to 10%.

Protein Kinase Assays

For determination of pp70S6K activity, cells were stimulated for 30 or 45 min and lysed in cold cell lysis buffer (73), and the lysates were cleared by centrifugation for 10 min at 13,000 × g. The immune-complex kinase assays were performed as described previously (80). S6 phosphotransferase activities were quantified with a Phosphorlmerager apparatus.

Immunoblot Analyses

Immunoblot analyses were performed as described (20). Aliquots of the clarified lysates containing 20–60 μg of cellular proteins were separated through SDS-7.5% PAGE and electrophoretically transferred to a nitrocellulose membrane. PP70 S6K was detected by the enhanced chemiluminescence method using rabbit polyclonal anti-pp70S6K antiserum as primary antibody.

PI3K Assay

PI3K assays were performed as described (81) with minor modifications. Cells stimulated with IL-2 (1,000 U/ml) for 7.5 min were lysed, and PI3K activities were immunoprecipitated with 4G10 antiphosphotyrosine antibody (a gift from B. Druker and T. M. Roberts, Harvard Medical School). The phosphotransferase assays were performed for 15 min at 30 C and terminated by addition of 200 μl of 1 N HCl. Phosphorylated phosphoinositides were separated by TLC on a silica gel 60 plate (Whatman, Inc., Clifton, NJ) that was impregnated with 1% potassium oxalate. CHCl3-methanol-4 M NH4OH (9:7:2) or n-propanol-2 N acetic acid (1:8:1) solution was used as developing solvent for TLC analysis. Phosphotransferase activities were quantified with a Phosphorlmerager apparatus.
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