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Different Early Effects of Tyrphostin AG957 and Geldanamycins on Mitogen-Activated Protein Kinase and p120^{cbl} Phosphorylation in Anti CD-3-Stimulated T-lymphoblasts

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ABSTRACT. AG957, a tyrphostin tyrosine kinase inhibitor, has been shown previously to inhibit p210$^{bcr-abl}$ phosphorylation with concurrent inhibition of p210$^{bcr-abl}$-expressing K562 cell growth (Kaur G and Sausville EA, *Anticancer Drugs* **7**: 815–824, 1996). To assess the specificity of the action of AG957, we have examined its effect in another tyrosine kinase-mediated system, anti CD-3-stimulated Jurkat T Acute Lymphoblastic Leukemia cells. We also compared the effects of AG957 with those of geldanamycin, which can disrupt tyrosine kinase signaling through binding to heat shock protein (hsp90), and two geldanamycin analogs, 17-amino-17-demethoxygeldanamycin (17AG) and 17-allylamino-17-demethoxygeldanamycin (17AAG). At concentrations found to produce 90% inhibition of Jurkat T-cell growth, AG957 within 4 hr of addition inhibited mitogen-activated protein (MAP) kinase activation and activity, as shown by a decreased anti CD-3-stimulated erk-2 mobility shift in lysates of treated cells and a decrease in the stimulated myelin basic protein peptide kinase activity in erk-2 immunoprecipitates, respectively. AG957 did not inhibit this activity when added directly to immunoprecipitates. Effects in cells were found to be accompanied by a decrease in the anti CD-3-stimulated phosphorylation of p120^{cbl}. Under conditions of a similar degree of growth inhibition, geldanamycin initially did not inhibit MAP kinase activation. Geldanamycin analogs did not decrease anti CD-3-induced cbl phosphorylation, but did reduce basal p120^{cbl} tyrosine phosphorylation. The action of AG957 occurred with an apparent shift of several tyrosine-phosphorylated proteins to apparent higher molecular weights, which also did not occur with the geldanamycins. These results suggest that growth inhibition by AG957 can alter tyrosine kinase signaling systems unrelated to p210$^{bcr-abl}$ with a prominent early effect on MAP kinase activation in T-lymphoblasts. AG957 and geldanamycin affect tyrosine kinase signaling by distinct mechanisms. BIOCHEM PHARMACOL **57**;3:281–289, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. tyrphostin; AG957; geldanamycin; cbl; MAP kinase

Tyrosine kinase inhibitors may inhibit signal transduction pathways important to specific cell types. Such inhibitors have been used to elucidate the involvement of tyrosine phosphorylated substrates within signal transduction cascades. AG957 is a tyrphostin tyrosine kinase inhibitor (see Fig. 1), originally modeled after the naturally occurring compound erbstatin [1]. In contrast to several known tyrosine kinase inhibitors that are ATP antagonists, such as quercetin and genistein, tyrphostins were designed to be antagonists of potential protein substrates. In certain cases, tyrphostins do compete with substrate and are noncompetitive with ATP [2, 3].

Geldanamycin is a benzoquinoid ansamycin antibiotic that has been studied extensively as an antitumor agent and as an inhibitor of tyrosine kinases [4]. It has been shown to reverse Src-induced transformation in cultured cells, as well as that induced by other oncogene tyrosine kinases [5]. However, geldanamycin does not inhibit kinase activity directly [6]. Recent studies have shown that this compound binds to the 90 kDa heat shock protein molecular chaperone (hsp90)†, inhibiting the capacity of hsp90 to form complexes [7, 8] with client proteins. These include several tyrosine kinases. Thus, the inhibition of tyrosine kinase activity by geldanamycins is indirect and potentially nonspecific. Geldanamycin has a quinone moiety as an integral

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† *Abbreviations:* hsp90, heat shock protein 90; G, Geldanamycin; 17AG, 17-amino-17-demethoxygeldanamycin; 17AAG, 17-allylamino-17-demethoxygeldanamycin; MAP kinase, mitogen-activated protein kinase; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

part of its ring system (Fig. 1); AG957 has the potential for oxidation to a quinone moiety.

Chronic myeloid leukemia (CML) is a neoplasm whose pathogenesis is causally linked to the expression of the $p210^{bcr\text{-}abl}$ tyrosine kinase [9]. This protein arises from a chimeric gene formed by translocation of the chromosome 9 segment bearing the c-*abl* tyrosine kinase gene to the *bcr* region on chromosome 22. Initial studies have demonstrated that certain tyrphostins could differentiate human K562 cells, which express the $p210^{bcr\text{-}abl}$ kinase [10]. Other studies have shown that among tyrphostins structurally related to lavendustin A and piceatannol, AG957 completely inhibits $p210^{bcr\text{-}abl}$ kinase activity and potently inhibits K562 cell growth [11]. Whether growth inhibition is a direct result of $p210^{bcr\text{-}abl}$ kinase inhibition or results from effects on additional substrates "downstream" of bcr-abl has not been clarified. To address the capacity of the tyrphostin AG957 to act on cellular signaling systems not involving $p210^{bcr\text{-}abl}$, we elected to examine the effect of AG957 and congeners on Jurkat T Acute Lymphoblastic Leukemia (ALL) cells, a leukemia cell line that does not express $p210^{bcr\text{-}abl}$ but clearly responds to various stimuli with increased tyrosine kinase activity [12, 13]. An additional goal of the current studies is to consider the hypothesis that AG957 and geldanamycin could affect common targets owing to their shared capacity to function as quinones, in the case of AG957 after oxidation. Nucleophilic attack on susceptible sulfhydryl residues in certain tyrosine kinases has been associated with inactivation or loss of kinase activity [14].

Activation of T-cells occurs through a complex biochemical cascade initiated by the binding of an antigen to the T-cell receptor, resulting in the stimulation of several effector functions including cell proliferation, differentiation, and lymphokine secretion [15, 16]. The T-cell receptor is a multi-subunit complex comprised of Ti α- and β-chains (associating with CD-3), γ-, δ-, and ϵ-chains, and a ζ-chain containing homodimer [14, 17–19]. The ligand binding domain consists of the α- and β-chains and recognizes the antigenic peptide bound to a major histocompatibiltiy complex (MHC). The T-cell receptor possesses no intrinsic kinase activity, but it initiates downstream activities by association with the CD-3 complex [12, 20]. The CD-3 and ζ-chain subunits, which are responsible for activation of cytoplasmic protein tyrosine kinases, initiate the transduction process.

c-*cbl* is a protocogene originally identified as a cellular homolog of the transforming protein expressed by the murine Cas NS-1 retrovirus [21]. It was later identified as a 120 kDa tyrosine phosphoprotein, which serves as one of the earliest tyrosine kinase substrates upon T-cell stimulation [22]. The v-*cbl* oncogene product is a severely truncated form of the cellular homolog, containing only the N-terminal 355 amino acids of c-*cbl*. It possesses a potential nuclear localization signal, but has been demonstrated in both the nucleus and the cytoplasm [23]. Recent studies have shown c-*cbl* to be tyrosine phosphorylated in response to the activation of a number of signaling pathways [24–26], including, interestingly, $p210^{bcr\text{-}abl}$.

We address in this paper the capacity of AG957, originally described as a $p210^{bcr\text{-}abl}$-directed kinase inhibitor, and geldanamycin congeners to interfere with T-cell signal transduction from T-cell antigen receptor. We demonstrated that AG957 can clearly block MAP kinase activation elicited by T-cell antigen receptor activation, and also inhibit c-cbl phosphorylation. We contrasted this action to those of a series of geldanamycin analogs, which appear to affect tyrosine kinase signaling by a distinct mechanism.

MATERIALS AND METHODS

Cell Culture, Growth Assay, and T-Cell Receptor Stimulation

Jurkat (clone E6-1 human acute T-cell leukemia; ATCC) cells were maintained in RPMI 1640 medium with 2 mM glutamine, 100 μg/mL of streptomycin and 100 U/mL of penicillin, and 10% fetal bovine serum. Cells (2×10^3 cells/well) were incubated with drugs in 200 μL of medium in 96-well plates. The growth of Jurkat T-cells compared with that of vehicle-treated cells was quantitated after 6 days by MTT dye reduction [27].

To assess the effects of drugs on T-cell antigen receptor stimulation, exponentially growing cells were resuspended in medium (1×10^6 cells/mL) 18–24 hr prior to the experiment, where they remained viable and continued to grow. Immediately prior to drug addition, cells were resuspended in medium at 15×10^6 cells/mL, a cell density necessary for optimal protein content for immunoprecipitation and western blot analysis. AG957, geldanamycin, and analogs were added to the cell suspension as indicated, and incubated at 37° for 4 hr. Then Aliquots (1 mL) were transferred to 1.5-mL tubes, to which was added vehicle or 1 μg/mL of anti CD-3 antibody, followed by shaking for 30 min at 37°. Cells were pelleted (14,000 *g* for 12 s), and pellets were lysed with 0.5 mL of Lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM $Na_4P_2O_7$, 100 mM NaF with the addition of 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride), vortexed, and centrifuged at 14,000 *g* for 15 min. Pellets were discarded, and the protein concentration of the supernatants was determined by the Bradford assay [28].

Immunoprecipitation and MAP Kinase Assay

Proteins were immunoprecipitated overnight using 500 μg cell lysate, 5 μg mouse monoclonal anti-phosphotyrosine (05-321 UBI), or 2 μg rabbit anti-cbl (SC-170 Santa Cruz Biotechnology) and pelleted with protein-A agarose beads (Oncogene Science). When immunoprecipitates were to be blotted, beads were washed twice with 500 μL HNTG buffer [200 mM HEPES, pH 7.5, 10% (v/v) glycerol, 0.1% Triton X-100, 150 mM NaCl, with addition of 1 mM

Na_3VO_4 immediately prior to use], and 30 μL of Laemmli sample buffer was added to each sample, which was then heated for 5 min at 95°. Samples were electrophoresed on a 4–20% SDS–PAGE gradient gel, and proteins were transferred to a PVDF membrane. MAP kinase was immunoprecipitated from 400 μg cell lysate, using 10 μg of anti erk-2 antibody (C-14 Santa Cruz) pelleted with protein-A agarose beads, and immunoprecipitates were washed twice in 0.5 mL kinase buffer immediately prior to kinase assay.

MAP kinase assays were performed with immunoprecipitates in a total volume of 40 μL of kinase buffer (12.5 mM β-glycerophosphate, 12.5 mM MOPS, pH 7.2, 0.5 mM EGTA, 7.5 mM $MgCl_2$, 0.05 mM NaF, 2 mM dithiothreitol, 2 mM $MnCl_2$) with 400 μM [γ-^{32}P]ATP (Dupont NEN; sp. act. 500–700 cpm/pmol), and 300 μM myelin basic protein (MBP) peptide substrate (12–125 UBI). The conditions of the assay were established to be linear with respect to time and amount of enzyme. Reactions were allowed to proceed for 20 min at 30° and were stopped by the addition of 10 μL of 5% acetic acid. After centrifugation at 14,000 *g* for 5 min, 30 μL of the supernatant was spotted onto a 1.5 cm^2 p81 paper (Whatman). Papers were washed five times in 5% acetic acid, once in acetone, and then counted by liquid scintillation spectrometry, and the picomoles of phosphopeptide produced per milligram protein per minute was calculated. Autophosphorylation was not observed in reactions that lacked substrate peptide.

Immunoblotting

Twenty-five micrograms of cell lysate protein was added to Laemmli sample buffer and heated at 95° for 5 min. Lysates were loaded onto a 20-cm 7.5% SDS–PAGE gel, electrophoresed, and transferred to PVDF membrane. The membrane was blocked with 3% BSA in TBST (20 mM Tris–HCl, pH 7.5, 0.9% NaCl, 0.05% Tween-20) for a minimum of 1 hr at room temperature. After blocking, the blots were probed with 1 μg/mL of anti erk-2 (C-14 Santa Cruz); 1 μg/mL of mouse monoclonal anti-phosphotyrosine (05-321 UBI); or 10 μg/mL of $p120^{cbl}$ (SC-170 Santa Cruz) in TBST. The blots were washed in TBST with 0.5% BSA and probed with 0.01 μg/mL of anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase. The blots were developed by chemiluminescence detection (ECL Detection Kit RPN 2209, Amersham).

$^{32}PO_4$-Labeled Tyrosine Phosphoproteins in Jurkat T-Cells

Jurkat T-cells (2×10^6 cells/mL) were resuspended in RPMI medium with 10% FBS, 10-mL aliquots were put in 100-cm plates, and drug or vehicle (DMSO) was added, as indicated, for 3 hr. Cells were collected, spun down, and resuspended in 3 mL of phosphate-free medium containing drug, as indicated. [^{32}P]-Orthophosphate was added (500 μCi/mL) for 1 hr at 37°. Cells were collected, spun down, and washed twice with PBS. Cells were lysed with 0.5 mL of Lysis buffer, vortexed, and immunoprecipitates formed, as described above, prior to electrophoresis. After drying the gel, labeled phosphoproteins were detected by PhosphorImaging (Molecular Dynamics Storm 860).

RESULTS

Effect of Inhibitors on Jurkat T-Cell Growth

To define the optimal concentration for drug use, we examined the effects of the tyrphostin AG957 (Fig. 1A), and of G, 17AG, and 17AAG (Fig. 1B). Jurkat cells were incubated for 6 days with each inhibitor. Panels C–F of Fig. 1 demonstrate that the IC_{90} values (the concentration of inhibitor necessary to produce 90% inhibition of cell growth) were approximately 25 μM for AG957, 0.1 μM for G, 10 μM for 17AG, and 50 μM for 17AAG.

Effect of AG957 on Stimulated MAP Kinase in Jurkat T-Cells

The ability of the tyrphostin AG957 to inhibit immunoprecipitated MAP kinase from cells exposed to drug for 4 hr was examined using 25 μM AG957. Anti CD-3 (1 μg/mL) stimulation for 30 min increased MAP kinase activity by 2.5-fold in vehicle-treated cells, compared with unstimulated cells (Fig. 2A). This difference in magnitude exceeded that of other samples stimulated for 60 min and was also greater than the stimulation observed after 3 or 5 min (data not shown). Figure 2A also demonstrates that pretreatment of Jurkat T-cells with 25 μM AG957 for 4 hr completely blocked the capacity of MAP kinase to respond to anti CD-3 after 30 min, and blunted the response of MAP kinase after a 60-min exposure to anti CD-3 ($P < 0.035$; Student's *t*-test). To assess whether AG957 can inhibit MAP kinase directly, drug was added directly to immunoprecipitates from untreated stimulated Jurkat T-cells. No inhibition was observed up to 50 μM drug (data not shown).

Under similar conditions of incubation and stimulation, 0.1 μM G did not inhibit MAP kinase activity when cells were stimulated with 1 μg/mL of anti CD-3 for 30 or 60 min (Fig. 2B). After 30 min of anti CD-3 stimulation, 17AG (10 μM) decreased MAP kinase activity by 40% and 17AAG (50 μM) by 45%.

Activation of MAP kinase is accompanied by phosphorylation of Y^{185} and T^{183} on erk-2 and Y^{204} and T^{202} on erk-1 [29, 30], resulting in a decrease in the electrophoretic mobility of $p42^{erk\text{-}2}$ [30, 31]. To correlate the decrease in enzymatic activity described above, we examined the effect of AG957 and geldanamycin congeners on the mobility of $p42^{erk\text{-}2}$. Pretreatment of cells with 25 μM AG957 substantially prevented the appearance of altered mobility of MAP kinase after stimulation (Fig. 3). A doublet $p42^{erk\text{-}2}$ band however, was still evident in lysates from cells exposed to 0.1 μM G, 10 μM 17AG, and 50 μM 17AAG. These observations are concordant with the effect on enzyme activity immunoprecipitated from whole cells. They suggest

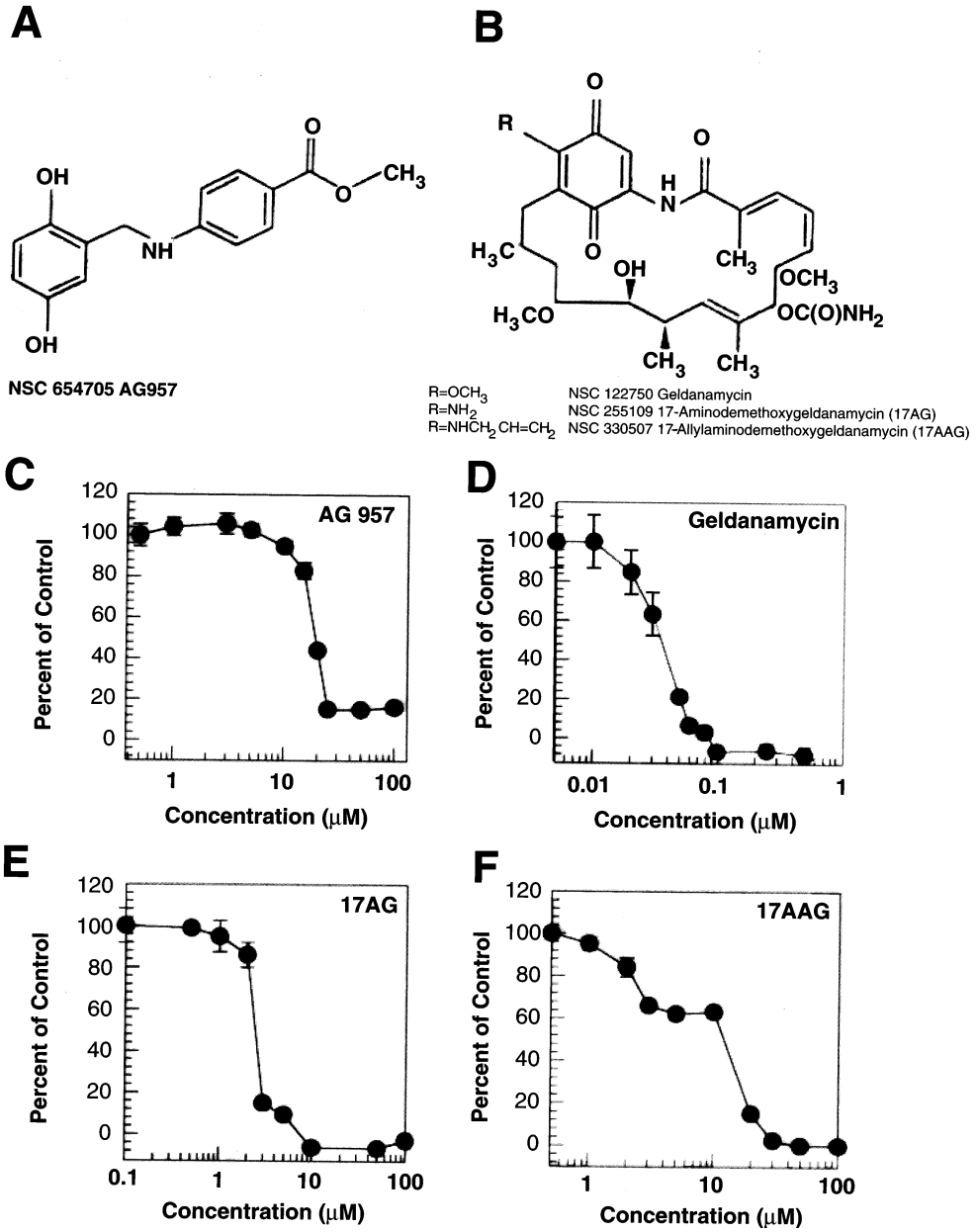

FIG. 1. Structures and growth inhibition. Structure of the tyrphostin AG957 (A) and geldanamycin congeners (B). In panels C–F, exponentially growing cells (2×10^3) were treated with the indicated concentrations of AG957 or geldanamycin congeners for 6 days. The inhibition of cell growth was determined by the MTT assay. Values are means ± SEM of three independent experiments.

that in concentrations of drug retarding growth to the same extent, AG957 has a major and prompt (within 4 hr) inhibition of MAP kinase activity in response to external stimuli, whereas G, 17AG, and 17AAG do not act to down-regulate efficiently MAP kinase activity after that period of drug exposure.

Effects of Inhibitors on ^{32}-PO$_4$-Labeled Jurkat T-Cells

The effect of AG957 on the tyrosine phosphorylation of proteins in Jurkat cells was examined using [^{32}P]orthophosphate labeling. Cells were labeled in the last hour of a 4-hr drug incubation, using inhibitors at respective IC_{90} concentrations. PhosphorImages of electrophoresed immunoprecipitated phosphotyrosines from labeled cells showed a decrease in overall tyrosine phosphorylation in cells exposed to each of the inhibitors (AG957, G, 17AG, 17AAG) (Fig. 4A). However, the pattern of AG957-treated cells differed from that of the geldanamycins in that there was a more prominent decrease in the phosphorylation of bands at approximately 120 and 220 kDa, and AG957 produced a slight increase in phosphorylation at approximately 35 kDa.

p120^{cbl} is a prominent component of T-cell signaling in response to antigen [22] and is a candidate 120 kDa target of AG957 action. Figure 4B shows that AG957 readily and essentially completely inhibited the phosphorylation of the p120 kDa cbl protein in comparison with control untreated

cells, suggesting cbl as a target of AG957. The geldanamycin compounds, in contrast, showed at best a slight decrease in the phosphorylation of $p120^{cbl}$ compared with that caused by AG957. Quantitation by PhosphoImager revealed an 18% reduction by G, 25% by 17AG, and 22% by 17AAG. Thus, although the geldanamycin compounds caused Jurkat cell growth inhibition similar to that caused by AG957 under these conditions, these experiments suggest an altered spectrum in their effect on tyrosine kinase targets on the part of the geldanamycins in comparison with AG957.

Effect of AG957 on Jurkat Phosphotyrosine Protein

As studies employing radiolabeled orthophosphate may preferentially assess tyrosine kinase proteins capable of autophosphorylation, or serving as substrates for other kinases, we examined phosphotyrosine mass in drug-treated cells under the same conditions employed for the MAP kinase activity experiments. Stimulation by anti CD-3 produced an increase in overall phosphorylation of tyrosine proteins compared with the unstimulated control (Fig. 5). This was accompanied by the development of phosphorylation not seen in control at ~30 and to a greater extent at ~20 kDa. This induced phosphorylation appears to be altered significantly in cells treated with AG957. In comparison with unstimulated control cells, AG957 altered the appearance of 30 and 120 kDa tyrosine phosphorylated protein, but also caused an apparent molecular weight shift of tyrosine phosphorylated proteins with an altered appearance above the 97 kDa marker. This is shown by the development of a darkened smear at >97 kDa in the phosphotyrosine blot. This effect of AG957 has been observed previously where components of the $p210^{bcr\text{-}abl}$ signaling system appear in higher molecular weight complexes, even in SDS–PAGE [11]. Geldanamycin congeners produced effects that were quite different in comparison to AG957. Geldanamycin after 4 hr (0.1 μM) did not produce a significant effect on the tyrosine phosphorylation in unstimulated or anti-CD-3-stimulated cells. 17AG and 17AAG showed a decrease in the overall mass of tyrosine-phosphorylated proteins in unstimulated cells, but did not appear to affect CD-3-stimulated increase in tyrosine phosphorylation of qualitatively similar proteins to that evoked by anti CD-3, e.g. between 46 and 120 kDa. 17AAG-treated cells, however, did show a decrease in tyrosine phosphorylation of proteins at both ~30 and ~20 kDa.

$p120^{cbl}$ was immunoprecipitated from Jurkat cells under the same conditions of drug treatment and stimulation. Consistent with the experiments shown in Fig. 4, a modest increase (9%; lane 1 vs lane 2) in phosphorylation was seen at 120 kDa when control cells were stimulated with anti CD-3 (Fig. 6A). Quantitation by PhosphorImager revealed that the basal tyrosine phosphorylation of p120 in unstimulated AG957-treated cells (lane 3) was decreased by 19% as compared with the unstimulated control (lane 1), with development of a darkened smear above 120 kDa protein,

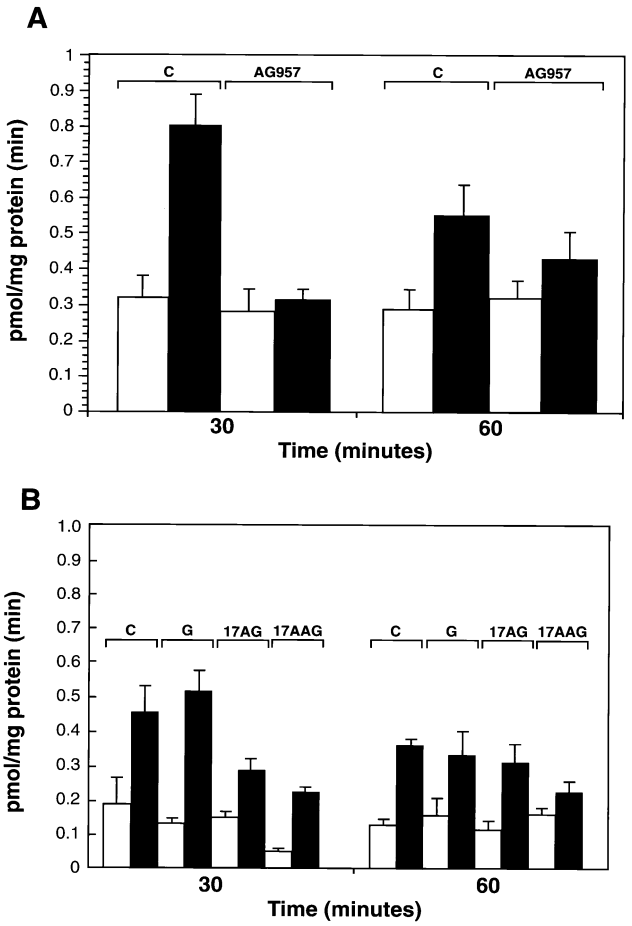

FIG. 2. Effects of drugs on MAP kinase activity from Jurkat cells. MAP kinase was assayed as pmol/min/mg protein of ^{32}P incorporated into MBP from lysate immunoprecipitated with anti erk antibody. (A) Cells were exposed to 25 μM AG957 or DMSO as drug vehicle (C) for 4 hr and then were stimulated with PBS (□) or 1 μg/mL of anti CD-3 (■) for 30 or 60 min, prior to immunoprecipitation of erk-2 activity. (B) Control cells were exposed to DMSO vehicle or 0.1 μM G, 10 μM 17AG, or 50 μM 17AAG, as indicated above each pair of bars, for 4 hr and then were stimulated with PBS (□) or 1 μg/mL of anti CD-3 (■) for 30 or 60 min prior to immunoprecipitation of erk-2 activity. In A and B, values (means ± SEM) are representative of three independent experiments performed in triplicate (AG957 vs vehicle and 17AAG vs vehicle, $P < 0.01$; 17AG vs vehilce, $P < 0.05$ by the Student–Newman–Kuel test).

similar to that seen in the phosphotyrosine blots in Fig. 5. In CD-3-stimulated cells, AG957 caused a 35% decrease in the tyrosine phosphorylation of $p120^{cbl}$ (lane 4) compared with the stimulated control (lane 2). This reduction in band size was again accompanied by several anti-cbl-reacting species of apparent higher molecular weight. Geldanamycin decreased the phosphorylation of $p120^{cbl}$ by 18% in unstimulated cells (lane 1 vs lane 5), and was unable to reduce the phosphorylation induced by anti CD-3 (lane 2 vs lane 6). The geldanamycin analogs greatly decreased the basal tyrosine phosphorylation state of $p120^{cbl}$, 17AG by 58% and 17AAG by 63%, respectively.

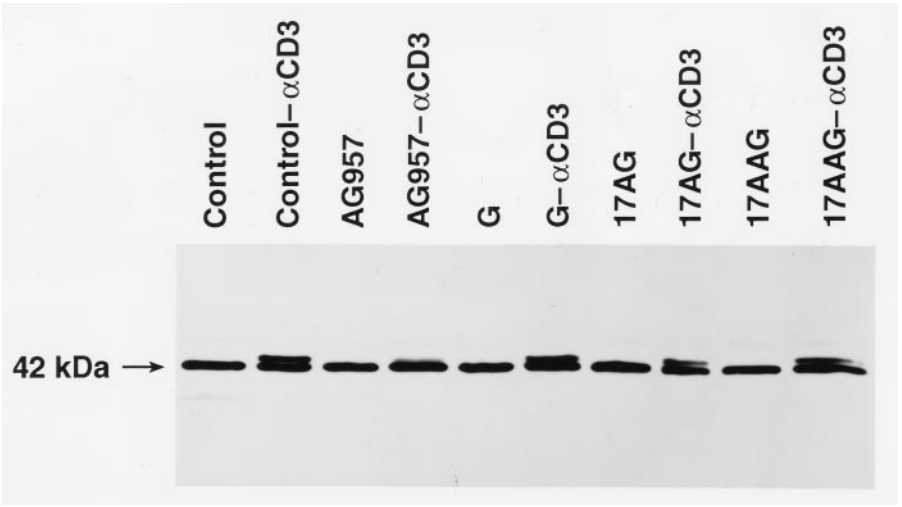

FIG. 3. Effects of AG957 and geldanamycin compounds on the "mobility shift" of MAP kinase. Jurkat T-cells were incubated with inhibitors at IC_{90} (25 μM AG957, 0.1 μM G, 10 μM 17AG, 50 μM 17AAG) or DMSO as control vehicle for 4 hr at 37°. Then cells were stimulated with 1 μg/mL of anti CD-3 for 30 min. Cell lysate proteins were separated by electrophoresis, transferred to PVDF membrane, and blotted with anti erk-2 antibody.

Despite the decrease in basal $p120^{cbl}$ phosphorylation, there was an insignificant effect of G and 17AG on anti CD-3-stimulated tyrosine phosphorylation of c-cbl (17AAG caused a 13% decrease in tyrosine phosphorylation; lane 2 vs lane 10).

When immunoprecipitated cbl was blotted with anti-cbl, the content of native $p120^{cbl}$ protein appeared to be altered by AG957 in both unstimulated and stimulated cells (Fig. 6A). There was a reduction of the band intensity at 120 kDa, accompanied by the appearance of a higher molecular weight species above 120 kDa. There was no effect on the mass of $p120^{cbl}$ after exposure to any geldanamycin analog in either anti CD-3-unstimulated or -stimulated cells (Fig. 6B).

DISCUSSION

Both the tyrphostin AG957 and the geldanamycins are tyrosine kinase signaling inhibitors. The results of this study show the tyrphostin AG957 to be an effective inhibitor of T-cell antigen-originated signal transduction and cell growth in Jurkat T-cells, but affecting tyrosine kinase signaling differently from the geldanamycins. At concentrations shown to inhibit 90% Jurkat T-cell growth, AG957 inhibited MAP kinase activation by anti CD-3 stimulation (Figs. 2A and 3) with a decrease in the stimulated increase in tyrosine phosphorylation of $p120^{cbl}$ (Figs. 4 and 6). At concentrations affecting cell growth to the same extent, geldanamycin and analogs showed no or less potent early MAP kinase inhibition in CD-3-stimulated cells (Fig. 2B), and the geldanamycin analogs decreased $p120^{cbl}$ tyrosine phosphorylation in the basal, non-CD-3-stimulated state only, with little effect on $p120^{cbl}$ tyrosine phosphorylation induced in anti-CD-3-stimulated cells (Fig. 6). In contrast to the effects produced by the geldanamycin compounds, AG957 action was accompanied by evidence of an altered physical state of target proteins, including $p120^{cbl}$.

These results reinforce the conclusion that although initial interest in tyrphostin AG957 was related to action on $p210^{bcr\text{-}cbl}$ [11], the drug is clearly not $p210^{bcr\text{-}cbl}$ specific but is able to affect other tyrosine kinase signaling systems. The protooncogene product $p120^{cbl}$ has been shown to

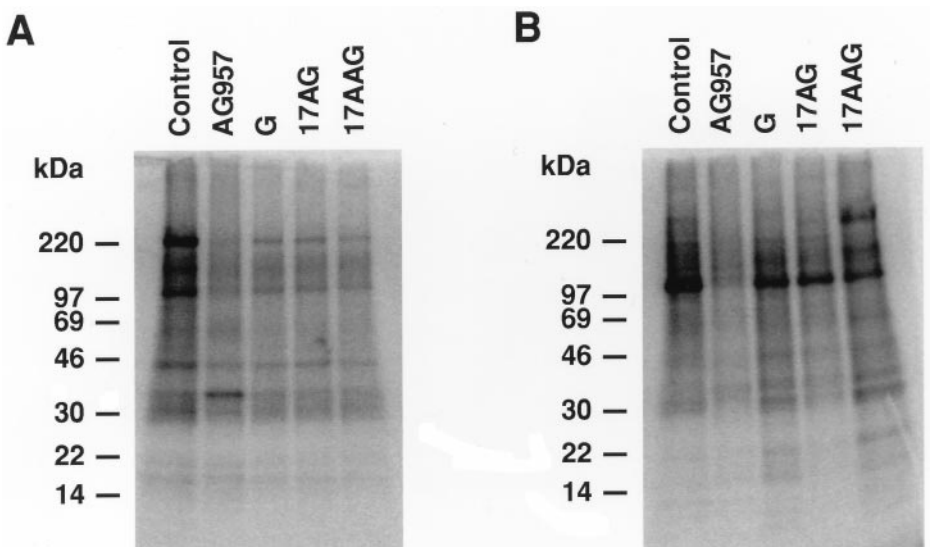

FIG. 4. Effect of inhibitors on $^{32}PO_4$ labeling of Jurkat proteins. Jurkat cells were incubated with inhibitors at IC_{90} (25 μM AG957, 0.1 μM G, 10 μM 17AG, 50 μM 17AAG) or DMSO as control vehicle for 4 hr at 37° and labeled in the last hour with $^{32}PO_4$. Phosphotyrosine proteins (A) or $p120^{cbl}$ (B) were then immunoprecipitated from labeled cells, separated by electrophoresis, and detected by PhosphorImaging.

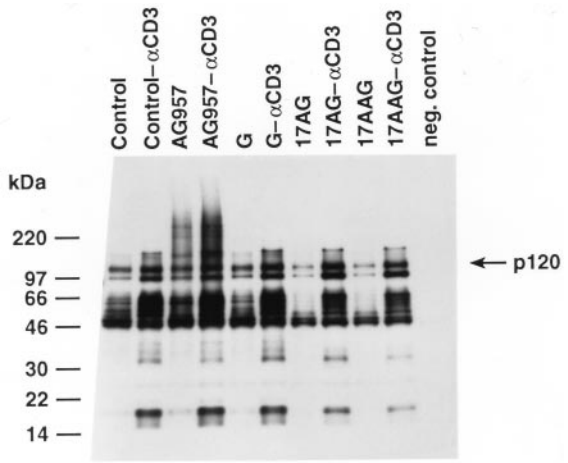

FIG. 5. Effect of AG957 and geldanamycin compounds on anti CD-3-stimulated tyrosine phosphoproteins. Jurkat T-cells were incubated with inhibitors at IC_{90} (25 μM AG957, 0.1 μM G, 10 μM 17AG, 50 μM 17AAG) or DMSO as control vehicle for 4 hr at 37°. Then cells were stimulated with 1 μg/mL of anti CD-3 for 30 min. Phosphotyrosine proteins were immunoprecipitated from cell lysates, separated by electrophoresis, transferred to PVDF membrane, and then blotted with anti-phosphotyrosine antibody. Negative controls with cell lysate, and without immunoprecipitating antibody, were included. The effect of prior exposure to inhibitors of tyrosine phosphorylation was compared with control cells in both anti CD-3-stimulated and antibody vehicle control conditions.

undergo rapid tyrosine phosphorylation in response to T-cell receptor (TcR) stimulation. Furthermore, this 120 kDa cytoplasmic protein, known as one of the earliest tyrosine kinase substrates upon T-cell stimulation, has also been implicated in several tyrosine kinase-linked pathways [23–26]. Notably, this has also included abl-mediated tumor

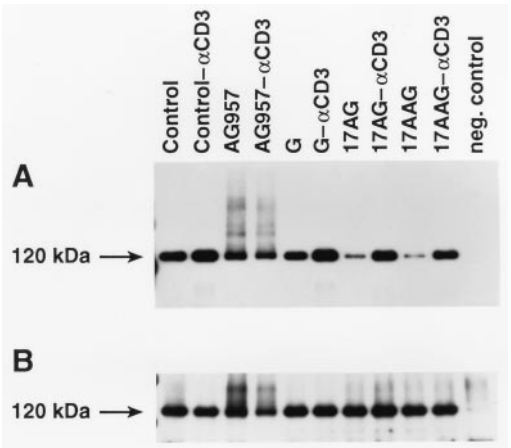

FIG. 6. Effects of AG957 and geldanamycins on $p120^{cbl}$ tyrosine phosphorylation. Jurkat T-cells were incubated with inhibitors (25 μM AG957, 0.1 μM G, 10 μM 17AG, 50 μM 17AAG) or DMSO as control vehicle for 4 hr at 37°. Then cells were either stimulated or not stimulated with 1 μg/mL of anti CD-3 for 30 min. $p120^{cbl}$ was immunoprecipitated from cell lysates, separated by electrophoresis, and transferred to PVDF membrane. Membranes were blotted with anti-phosphotyrosine (A) or anti-cbl (B). Negative controls contained cell lysate in the absence of precipitating antibody.

induction. $p120^{cbl}$ has been shown to bind *in vitro* with the SH3 and SH2 domains of lck, fyn, src, lyn, and grb2 [23, 25]. $p120^{cbl}$ has also been demonstrated to associate with adaptor proteins, such as nck and crk [24]. Its role may be to serve as a "scaffold" upon which tyrosine kinases and their adaptors are assembled. Our findings suggest that AG957 prominently affects pathways converging on $p120^{cbl}$. This would also be concordant with the prominent previously demonstrated [11] influence of the drug on CML cell growth (by affecting $p210^{bcr\text{-}abl}$), as well as T-cell antigen-related signaling, shown here. Further experiments to dissect the susceptibility of specific $p120^{cbl}$-modulated events to AG957 should be of interest.

In addition to inhibition of tyrosine phosphorylation of target proteins, such as $p120^{cbl}$, the tyrphostin also acts to alter the native state of its target proteins, as shown in the western blots in Figs. 5 and 6. When total phosphotyrosine-containing proteins, as well as $p120^{cbl}$, were immunoprecipitated from AG957-treated cells, the western blots produced from the immunoprecipitates consistently contained a characteristic darkened smear in the high molecular weight range of the blots. This characteristic had also been noted in immunoprecipitated $p210^{bcr\text{-}abl}$ from AG957-treated K562 cells, and was ascribed to potential covalent modification of the target molecule through a cross-linking mechanism [11]. A similar effect was also reported on the part of erbstatin analogs in normal and neoplastic cells [32]. The potential involvement of several different proteins in this cross-linking mechanism would likely produce several complexes of varying size in the high molecular weight range.

Although AG957 possesses the potential for oxidation to a quinone, similar to a structural feature of the geldanamycins, little similarity is apparent in the cellular effects of these two types of compounds. At the same AG957 concentrations that produce complete cell growth inhibition, the tyrphostin rapidly inhibits MAP kinase activation and cbl phosphorylation. In contrast, a rapid, prominent effect on MAP kinase signaling was not found with the geldanamycin compounds under similar conditions. Also, while AG957 affected basal and anti CD-3-stimulated $p120^{cbl}$ phosphorylation, geldanamycin displayed most prominent early effects on basal $p120^{cbl}$ tyrosine phosphorylation, with little effect on anti CD-3-stimulated events.

Recent studies have shown that geldanamycin acts by binding to the 90 kDa heat shock protein, with disruption of various hsp90 complexes [7] including those found with several tyrosine kinases and transcription factors [33]. Benzoquinoid ansamycins had been reported previously to inhibit CD-3 T-cell signaling through the inhibition of protein tyrosine kinases (PTK) [34]. However, in these studies the induced inhibition was shown to require a 12- to 16-hr pretreatment of T-cells with inhibitor to obtain an 85–95% reduction of the TcR-induced increase of substrate tyrosine phosphorylation. Under similar conditions of prolonged incubation, geldanamycin depletes c-raf [35] and inhibits MAP kinase [36, 37]. This downstream inhibition

was shown to be due to disruption of the hsp90–Raf-1 complex [36, 38]. Our experiments emphasize that a briefer period of exposure to concentrations of geldanamycin leading to growth inhibition was ineffective in decreasing MAP kinase activation and activity, while clearly inhibiting global tyrosine phosphate incorporation (Fig. 4A).

The present results encourage further studies to elucidate the mechanism of geldanamycin congener effects on $p120^{cbl}$-mediated signaling. In particular, association of $p120^{cbl}$ with lck, fyn, lyn, and grb2 [23, 24] may be disrupted by geldanamycin congeners in a way that can be related to effects on hsp90 association with these target proteins. The $p120^{cbl}$, a readily definable target in T-cells of geldanamycin congener action, would be an important potential surrogate marker of drug effect, as geldanamycin congeners are candidates for introduction into clinical trial as anti-proliferative agents. Conversely, greater specificity of AG957-like molecules may arise by considering aspects of its molecular interaction with $p120^{cbl}$-associated signaling molecules.

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