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Altered physical state of p210$^{bcr-abl}$ in tyrphostin AG957-treated K562 cells

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AG957 is a tyrosine kinase antagonist which prior studies had shown inhibits p210$^{bcr\ abl}$ tyrosine kinase activity and K562 chronic myelogenous leukemia cell growth. We report here the effects of AG957 on the physical state of p210$^{bcr\ abl}$ and its signaling adapter molecules Shc and Grb2 in K562 cells. After exposure to AG957, the amount of tyrosine-phosphorylated native p210$^{bcr\ abl}$ decreases, with the appearance of a high molecular weight (> 210 kDa) p210$^{bcr\ abl}$. This effect is seen after [^{32}P]orthophosphate and [^{35}S]methionine labeling. Material suggesting the involvement of p210$^{bcr\ abl}$ in high molecular weight complexes also appears using anti-Shc, anti-Grb2 and anti-phosphotyrosine antibodies. AG957 also acts *in vitro* to shift native p210$^{bcr\ abl}$ in anti-p210$^{bcr\ abl}$ or anti-Grb2 immunoprecipitates to higher molecular weight forms under conditions where the drug can also act as an antagonist of p210$^{bcr\ abl}$ autokinase activity. Incubation with dithiothreitol inhibits the appearance of > 210 kDa forms of p210$^{bcr\ abl}$ in the *in vitro* reaction. These results lead to the hypothesis that AG957 does not act simply as a reversible tyrosine kinase antagonist, but can alter the normal amounts and physical associations of molecules important in tyrosine kinase signaling. These effects likely reflect covalent cross-links induced by the drug.

Key words: Chronic myelogenous leukemia, Grb2, tyrosine kinase inhibition.

Introduction

The pathogenesis of chronic myelogenous leukemia (CML) has been linked to a reciprocal translocation which fuses exons from the Bcr gene on chromosome 22 with exons of the c-*abl* proto-oncogene from chromosome 9. The Philadelphia chromosome is the cytogenetic marker for this event. The resulting reading frame encodes a p210$^{bcr-abl}$ chimeric oncoprotein, with constitutively activated tyrosine kinase activity.[1–4] p210$^{bcr\ abl}$ is localized to the cytoplasm and has transforming potential in contrast to the normal c-*abl* protein.[5] Inhibitors of p210$^{bcr\ abl}$ tyrosine kinase activity may be of value in the treatment of CML and in patients with acute lymphocytic leukemia (ALL) who express a different p185$^{bcr\ abl}$ fusion protein but with similar or perhaps somewhat enhanced transforming potency.

Recent studies have demonstrated that growth factor receptor-linked tyrosine kinases can activate a signaling pathway through Ras. The Grb2 protein, composed of a single SH2 domain flanked by two SH3 domains, can mediate the physical association of certain receptor tyrosine kinases to mSos1 in mammals[6] and in *Drosophila*[7–12] by non-covalent binding of phosphorylated tyrosines. mSos1 induces GDP release from Ras, which then can bind GTP and become activated. Several reports have identified specific tyrosine phosphate sequences in receptor tyrosine kinases[6,8,10] which mediate direct binding of the Grb2 SH2 domain. Grb2 can also interact with the kinase signaling pathways indirectly via Shc[13,14] or PTP1/Syp.[15] Although Grb2 associates with activated tyrosine kinases, it is itself not an efficient substrate for tyrosine phosphorylation in either *Drosophila* or mammalian cells.[6,11,14]

The mammalian Shc oncogene encodes three isoforms; two widely expressed proteins of 46 and 52 kDa, and a less widely expressed 66 kDa isoform. These proteins possess a tyrosine at position 317, which is a substrate for a variety of non-receptor and receptor tyrosine kinases, and which binds to the SH2 domain of Grb2. Shc itself also possesses a C-terminal SH2 domain and a more central glycine/proline-rich region.[16] Shc appears to function as a bridge between the Grb2–mSos complex and non-receptor tyrosine kinases by binding of Grb2–mSos to the phosphorylated Y317 of Shc.[16,17] Recent studies on mitogenic signaling by fibroblast growth factor,[18] and insulin, insulin-like growth factor 1 and epidermal growth factor[19] have suggested that tyrosine phosphorylation of Shc is essential to mitogenic signaling, and it has been shown that tyrosine phosphorylation of Shc is crucial for the transforming ability of v-*src* and v-*fps*.[13,20] Thus, Grb2 and Shc

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are implicated in downstream signaling from tyrosine kinases in many different biological systems.

The $p210^{bcr\ abl}$ protein can form a specific complex with Shc and with Grb2, as revealed in studies with three different murine cell lines transfected with a $p210^{bcr\ abl}$ expression vector.[21] In these cells expressing the $p210^{bcr\ abl}$, Shc proteins were highly phosphorylated. The constitutive tight association of Shc and Grb2 in $p210^{bcr\ abl}$ transformed cells involved the presence of $p210^{bcr\ abl}$ in the same complex.[21] In naturally occurring K562 CML cells, the existence of $p210^{bcr\ abl}$ Grb2 and $p210^{bcr\ abl}$–Shc Grb2 complexes[21] also suggest that Grb2 and Shc are involved in Bcr–abl tyrosine kinase signaling.

Tyrphostins represent a class of synthetic tyrosine kinase antagonists modeled by Levitzki *et al.*[22] on the natural product erbstatin.[23] Four classes of tyrphostins have been described: benzenemalonitrile, *S*-aryl benzenemalonitrile, bisubstrate quinoline and lavendustin-A-like compounds.[22] In our initial efforts to discern a tyrosine kinase inhibitor as a lead structure directed against $p210^{bcr\ abl}$, AG957 was found to inhibit $p210^{bcr\ abl}$ autokinase activity in anti-$p210^{bcr\ abl}$ immunoprecipitates, and to decrease $p210^{bcr\ abl}$ phosphorylation in living K562 cells at concentrations and durations of exposure which also inhibit cell proliferation.[24,25] In this report we extend these observations by demonstrating that AG957 causes gross alteration of the physical state of the $p210^{bcr\ abl}$, Shc and Grb2 complexes, with decreased tyrosine phosphorylation of Shc. These results reveal that AG957 represents a class of tyrphostins that can decrease the normal level and physical association of molecules important in tyrosine kinase signaling. Our findings are consistent with and suggest an antiproliferative mechanism where AG957 induces the formation of covalent adducts involving $p210^{bcr\ abl}$ and its associated signaling molecules.

Materials and methods

Antibodies

A mouse monoclonal antibody raised against a synthetic peptide for Bcr protein was purchased from Oncogene Science (Uniondale, NY). Mouse anti-phosphotyrosine monoclonal antibody (clone 4G10) and rabbit polyclonal anti-Shc antibodies were from Upstate Biotechnology (Lake Placid, NY). Affinity-purified anti-Grb2 polyclonal antibody (raised against the entire protein) was from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit anti-mouse secondary antibody was from Pierce Biotech (Rockford, IL).

Cells and reagents

The K562 cell line was obtained from ATCC (Rockville, MD). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin. All cultures were maintained at 37°C in 5% CO_2. Tyrphostins AG957 and AG555 were provided by Dr L Malspeis (Laboratory of Pharmaceutical Chemistry, NCI). Stock drug solutions were prepared in 100% dimethylsulfoxide (DMSO) and maintained at −70°C in the dark.

Immunoprecipitation and immunoblotting

Cells were washed twice in phosphate buffered saline and were lysed in PLC lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride and 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged at 100 000 *g* for 15 min and the supernatant collected. Protein concentrations of the clarified supernatants were determined by the Bradford Protein Assay,[26] and the same quantity of protein was immunoprecipitated from control (vehicle-treated) and drug-treated cells. Cell lysates were incubated and gently rocked at 4°C for 4 h or overnight with antibodies. Immune complexes were collected by incubation with Protein A Sepharose beads (Pharmacia, Piscataway, NJ) and immune complexes (beads) were washed three times with HNTG buffer [20 mM HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mM NaCl and 1 mM sodium orthovanadate] to remove unbound material. Bound proteins were boiled for 5 min in 2 × SDS sample buffer (63 mM Tris HCl, pH 6.8; 10% glycerol; 2% SDS; 0.0025% bromophenol blue and 0.72 mM 2-mercaptoethanol) prior to SDS PAGE.[27] Proteins were resolved on 4–20% (gradient) mini gels from Novex Experimental Technology (San Diego, CA).

After SDS PAGE, proteins were electrophoretically transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) at 250 mA for 4 h at 4°C using 2 × Tris–glycine

transfer buffer (50 mM Tris, 384 mM glycine), 20% methanol and 0.1% SDS. Residual binding sites on the membrane were blocked by incubation in TTS (20 mM Tris, pH 7.4, 0.9% NaCl and 0.05% Tween 20) containing 3% BSA overnight at 4°C or for 4 h at room temperature. Blots were incubated for 1–1.5 h with TTS containing 0.3% BSA with 1 μg/ml of mouse anti-P-Tyr, 2 μg/ml of mouse anti-Grb2 or 1 μg/ml of rabbit anti-Shc antibody. Blots were washed three times for 6 min each with TTS containing 0.3% BSA and probed with a 1:1000 dilution of rabbit anti-mouse antibody or directly with [^{125}I]Protein A (Amersham, Arlington Heights, IL) in blocking solution. Proteins were visualized by autoradiography (exposed for 1–2 days at −70°C) or as indicated in figure legends.

Metabolic labeling

Cells were washed once with RPMI 1640 lacking either phosphate or methionine. Metabolic labeling was carried out in phosphate-free or methionine-free medium containing 5% dialyzed serum. Labeling was initiated by addition of 1 mCi/ml [^{32}P]orthophosphate (Amersham) for the last hour of drug exposure or cells were labeled with 0.5 mCi/ml of [^{35}S]methionine (ICN, Irvine, CA) for 90 min followed by removal of the label and incubation with drugs for 4 h at 37°C. Cells were washed three times with PBS, and were lysed and immunoprecipitated with antibodies as described earlier.

In vitro auto-phosphorylation reaction

The p210$^{bcr\ abl}$ protein immunoprecipitates were washed twice with HNTG buffer. Immunoprecipitates were washed once with 50 mM Tris (pH 7.0) and distributed to each reaction in 20 μl of 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0)–20 mM $MnCl_2$. Then, 10 μl of the appropriate tyrphostin (four times the desired concentration) from working stock solution prepared in 20 mM PIPES (pH 7.0)–20 mM $MnCl_2$ was added to each reaction tube. Reactions were initiated with the addition of 10 μl of [γ-^{32}P]ATP (10 μCi per sample, 3000 Ci/mmol; Amersham), incubated for 20 min at 30°C, stopped by addition of 10 μl of 5 × SDS sample buffer, heated at 100°C for 5 min, and analyzed on 4–20% SDS–PAGE and by autoradiography.

To assess the effect of drugs added *in vitro* on the state of p210$^{bcr\ abl}$ in the immune complexes, K562 cells were labeled with ^{32}P as described above, lysed in PLC buffer and immune complexes containing ^{32}P-labeled p210$^{bcr\ abl}$ were collected on protein Sepharose beads and beads were washed four times with HNTG buffer. Immune complexes were divided into several tubes in 30 μl of PLC buffer in the presence of tyrphostin as described in the figure legends. In another set of experiments ^{32}P-labeled immune complexes were preincubated with various concentrations of DTT or lysine for 5 min prior to the addition of tyrphostin AG957. Reactions were carried out at 30°C for 30 min and reactions were stopped by addition of 10 μl of 4 × SDS sample buffer. Samples were heated at 100°C for 5 min and proteins were resolved on 4–20% gradient gels.

V8 digestion of proteins in gel slices

A modification of the method described by Cleveland *et al.*[28] was employed. Individual bands were cut out from gel and soaked for 30 min in a buffer containing 0.125 M Tris–HCl, pH 6.8, 0.1% SDS and 1 mM EDTA. Sample wells of 1.5 mm thick gel were filled with this buffer and each gel slice was pushed to the bottom of a well with a spatula. Spaces around the slice were filled with 10 μl of this buffer containing 10% glycerol and a given amount of *Staphylococcus aureas* protease from V8 (Pierce) was overlayed into each slot; electrophoresis was performed in the normal manner with the exception that the current was turned off for 30 min when bromophenol blue dye neared the bottom of the stacking gel.

Results

Effect of AG957 on p210$^{bcr\ abl}$ and associated proteins

Previous studies[25] have demonstrated that tyrphostin AG957 (Figure 1E) can inhibit cell growth (IC_{50} = 15 μM) in association with decreased intracellular phosphorylation of p210$^{bcr\ abl}$. In contrast, tyrphostin AG555 (Figure 1E) inhibits cell growth (IC_{50} = 9.2 μM) without influence on p210$^{bcr\ abl}$ function and indeed was recently shown to inhibit topoisomerase I with moderate potency.[29] In an effort to define how AG957 may specifically interfere with p210$^{bcr\ abl}$ signaling, we compared the effects of AG957 and AG555 on the tyrosine phosphorylation of p210$^{bcr\ abl}$ and its association with the 'adapter' molecules Grb2 and Shc. Cells were

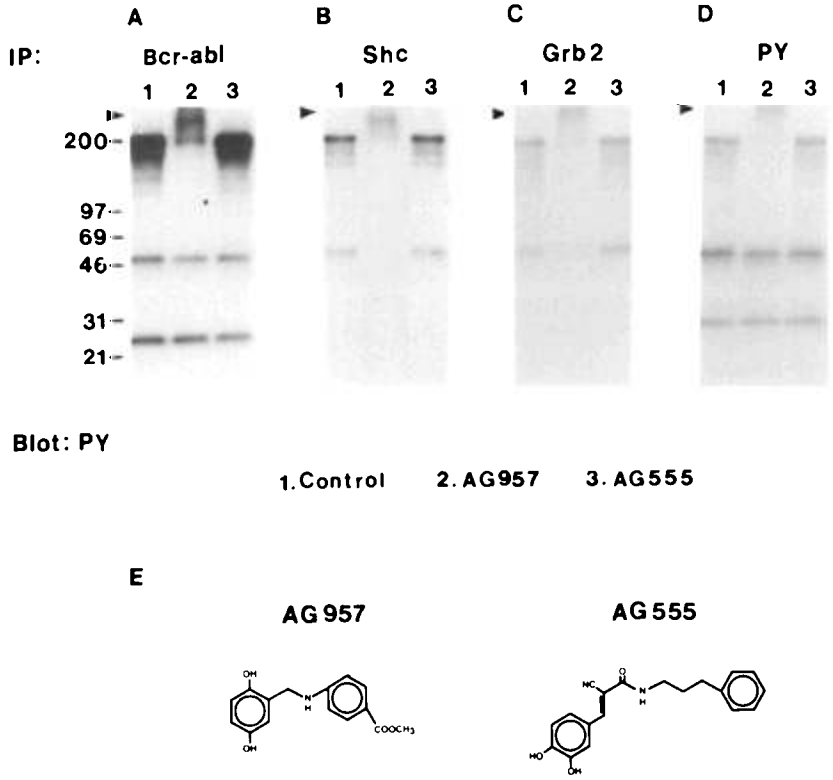

Figure 1. Effect of tyrphostins on tyrosine phosphorylated p210$^{bcr\ abl}$ and other adapter molecules. Cells were treated with drugs for 4 h followed by immunoprecipitation with anti-p210$^{bcr\ abl}$ (A), anti-Shc (B), anti-Grb2 (C) and anti-phosphotyrosine (D) antibodies. Following SDS PAGE and transfer of proteins to PVDF membranes, membranes were immunoblotted with anti-phosphotyrosine antibody (clone 4G10). Lane 1, cells not exposed to drug; lane 2, AG957-treated; lane 3, AG555-treated K562 cells. (E) Structures of AG957 and AG555.

exposed to AG957 or AG555 for 4 h, and the status of phosphotyrosine in anti-p210$^{bcr\ abl}$, Shc and Grb2 immunoprecipitates examined by Western blotting with an anti-phosphotyrosine antibody. A striking finding (Figure 1A) is the altered appearance of the p210$^{bcr\ abl}$ immunoprecipitate in AG957-treated cells. Specifically, there is a decrease in phosphotyrosine-containing material at the p210 position, with a smear (arrow) of phospho-tyrosine-containing material at $>$ 210 kDa. Both the native p210$^{bcr\ abl}$ and the smear containing phosotyrosine were competed by the immunizing peptide from Bcr abl (data not shown). Using an anti-Shc antibody, a decrease in the apparent tyrosine-phosphorylation of p52^{shc} is observed (Figure 1B) after exposure to AG957 but not AG555. Shc can be co-immunoprecipitated from human leukemia or Bcr abl-transfected cells with anti-p210$^{bcr\ abl}$ antibodies.[21] It is, therefore, noteworthy that again after exposure to AG957, phosphotyrosine-containing material at $>$ 210 kDa is present in immunoprecipitates formed with anti-Shc in AG957-treated cells, in contrast to the expected species at 210 kDa from untreated cells. Anti-Grb2 likewise (Figure 1C) co-immunoprecipitates, as expected, material at 210 kDa from control cells, but in AG957-treated cells again demonstrates material at $>$ 210 kDa, an effect also observed when both the precipitating and blotting antibody is directed against phosphotyrosine (Figure 1D).

As the smear of phosphotyrosine-containing material at $>$ 210 kDa appears despite the fact that samples were boiled and reduced prior to SDS PAGE, these findings suggest that AG957 can specifically (in comparison to AG555) cause the formation of a high molecular weight ($>$ 210 kDa) complex stable under the conditions of electrophoresis containing Shc, p210$^{bcr\ abl}$ and Grb2 in AG957-treated K562 cells. The specific effect on Shc tyrosine phosphorylation and the apparently altered physical state of p210$^{bcr\ abl}$ appears as early as 1 h after drug exposure, but by 24 h, a global decrease in phosphotyrosine-containing proteins can be observed (data not shown). Similar results were obtained when antibodies recognizing different epitopes from other suppliers were tested. These data lead us to propose that AG957 might be inducing the covalent associa-

tion of p210$^{bcr\ abl}$ protein molecules either to each other,[30] to cytoskeletal elements with which p210$^{bcr\ abl}$ associates[31] or to other elements in the p210$^{bcr\ abl}$-associated complex.[21,15,32]

Effect of AG957 on metabolically labeled proteins

To assess whether the effects of AG957 are evident even when detected directly, without Western blotting, K562 cells labeled with [^{35}S]methionine were lysed and the whole cell lysates electrophoresed. The resulting autoradiogram of the dried gel (Figure 2A) does not suggest alteration of the physical state of a great majority of cellular proteins by AG957. Immunoprecipitates formed with anti-Bcr–abl show a notable smear of radioactivity at > 210 kDa in comparison to either control or AG555-exposed cells, an effect also observed with anti-Shc, anti-Grb2 and anti-phosphotyrosine immunoprecipitates (Figure 2C–E). Notably, in the anti-phosphotyrosine immunoprecipitate (Figure 2E) no effect of the drug was evident with most protein bands, emphasizing the relative selectivity of the AG957 in altering the physical state of p210$^{bcr\ abl}$ and related signaling molecules. Immunoprecipitates of the same [^{35}S]methionine-labeled lysate with anti-Shc (Figure 2C) again revealed a specific effect of AG957 to decrease the labeling of a band at the 52 kDa region, concordant with a reduction in the mass of normally migrating Shc.

When proteins were immunoprecipitated and resolved on SDS–PAGE gels from [^{32}P]orthophosphate-labeled K562 cells, there was no decrease in global protein phosphorylation nor alteration of the appearance of most phosphoproteins after AG957 or AG555 exposure (Figure 3A). We again observed decreased phosphorylation of a protein at 210 kDa and a high molecular weight (> 210 kDa) species in anti-Bcr, anti-Shc, anti-Grb2 and anti-PY immunoprecipitates after AG957 exposure but not after exposure to AG555 (Figure 3B–E).

These experiments, therefore, are consistent with the idea that tyrphostin AG957 can induce the appearance of p210$^{bcr\ abl}$ and its associated signaling adapter molecules in an abnormal high molecular weight form in directly immunoprecipitated

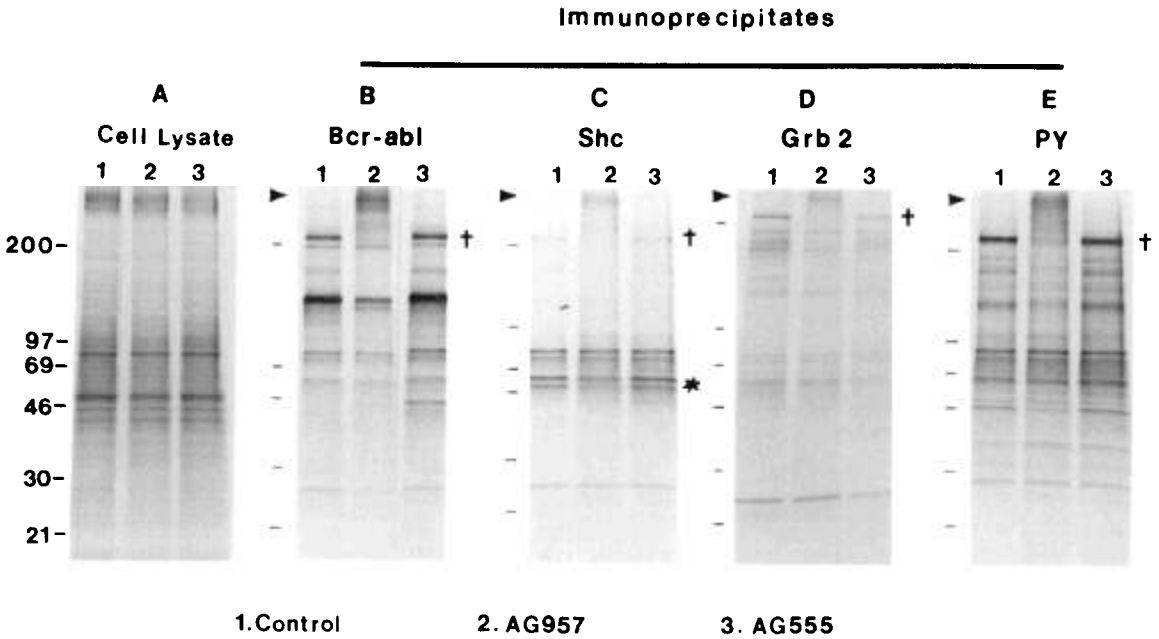

Figure 2. Effect of tyrphostins on [^{35}S]methionine-labeled cells. Cells were labeled in methionine-free media with [^{35}S]methionine (1 mCi/10 ml) for 90 min. Cells were washed three times with PBS followed by incubation in RPMI 1640 media containing AG957, AG555 or DMSO (vehicle control). After 4 h, cells were washed, lysed in PLC buffer and proteins were resolved as whole cell lysates (A), or were immunoprecipitated with anti-Bcr abl (B), anti-Shc (C), anti-Grb2 (D) or anti-PY (E, clone 4G10) antibodies. The horizontal lines to the left of each panel represent molecular weight markers; the arrowheads to the left of each panel designate the material migrating at > 210 kDa induced by AG957 treatment; the asterisks indicate the protein of 52 kDa whose labeling is diminished in AG957-exposed anti-Shc immunoprecipitates; the daggers indicate the position expected for native p210$^{bcr\ abl}$ protein. Lane 1, control; lane 2, AG957-treated K562 cells; lane 3, AG555-treated K562 cells.

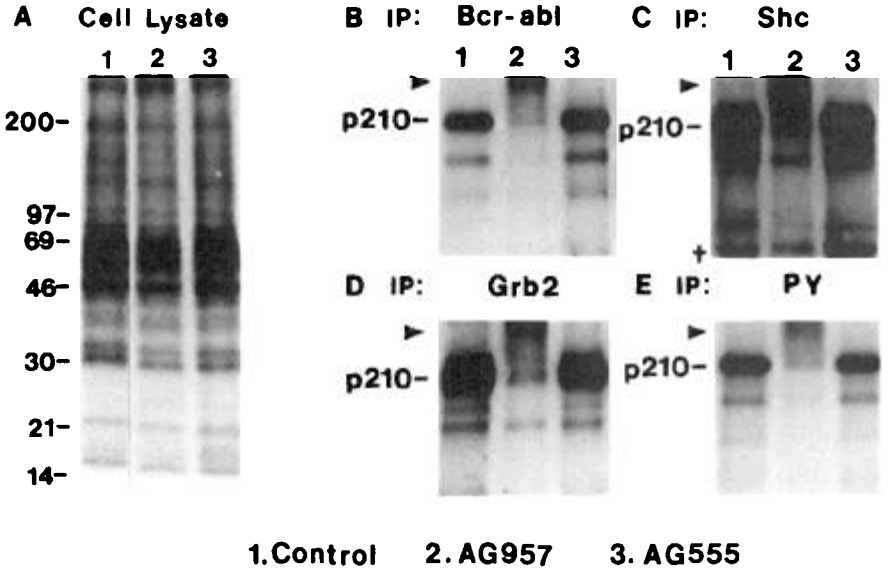

Figure 3. Effect of tyrphostins on [^{32}P]orthophosphate-labeled cells. Cells were exposed to AG957, AG555 or DMSO (vehicle control) for 4 h. The last hour of incubation was carried out in the presence of 1 mCi/ml [^{32}P]orthophosphate in phosphate-free media containing dialyzed serum. Cells were washed, lysed in PLC buffer and proteins were resolved as whole cell lysates (A), or were immunoprecipitated with (B) anti-Bcr, (C) anti-Shc [dagger shows the expected position of Shc in the indicated panel], (D) anti-Grb2 or (E) anti-phosphotyrosine antibodies. Lane 1, vehicle-treated cells; lane 2, AG957-treated K562 cells; lane 3, AG555-treated K562 cells.

metabolically labeled proteins (Figures 3 and 4) as well as proteins detected by immunoblot methods (Figures 1 and 2).

V8 protease patterns

To provide evidence that the > 210 kDa material in AG957-treated cells actually contained p210$^{bcr\ abl}$, we conducted protease mapping of the p210 material associated with Grb2 in vehicle-treated, non-drug-treated cells (Figure 4A) and the > 210 kDa material associated with Grb-2 in AG957-treated cells (Figure 4B). The similarity of the V8 protease digestion pattern is concordant with the presence of p210$^{bcr\ abl}$ in species of > 210 kDa in AG957-treated cells.

Effect of AG957 on size and kinase activity of p210$^{bcr\ abl}$ protein *in vitro*

As the results presented in Figures 1–4 provide evidence that in living K562 cells, AG957 alters the physical state of p210$^{bcr\ abl}$, we examined the effect exposure to AG957 has *in vitro* on immunoprecipitated p210$^{bcr\ abl}$ and on p210$^{bcr\ abl}$ co-immunoprecipitated with Grb2. Both unlabeled cells and cells metabolically labeled with [^{32}P]orthophosphate

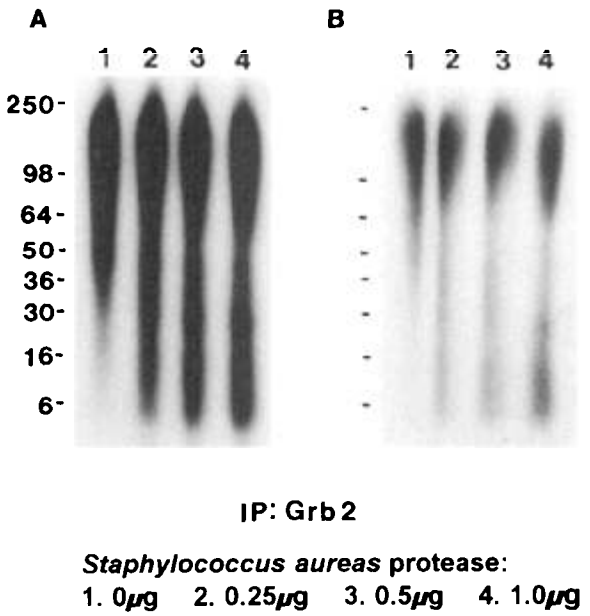

Figure 4. Protease digestion pattern. (A) Grb2 immunoprecipitates from [^{32}P]orthophosphate-labeled K562 cells were electrophoresed and the p210$^{bcr\ abl}$ protein was excised and digested with the indicated amount of V8 protease. (B) Grb immunoprecipitates from AG957-treated (25 μM), [^{32}P]orthophosphate-labeled K562 cells were electrophoresed and the material at > 210 kDa excised and digested with the indicated amount of V8 protease. Samples from A (control) and B (AG957-treated) were electrophoresed on the same gel.

were lysed, and immunoprecipitates formed with anti-Bcr or anti-Grb2 antibodies. In Figure 5(A) immunoprecipitates prelabeled with ^{32}P were exposed to various concentrations of AG957 or AG555 for 30 min at 30°C and proteins were resolved by SDS PAGE gel electrophoresis. AG957 causes a decrease in the electrophoretic mobility of p210$^{bcr\ abl}$ in a concentration-dependent manner (Figure 5A). This results in an apparent decrease in the amount of native p210$^{bcr\ abl}$ protein with increasing drug concentrations and an increase in a high molecular weight (> 210 kDa) smear formation. Similar results were obtained with ^{32}P-labeled Bcr–abl protein co-immunoprecipitated with anti-Grb2 (Figure 5C). AG555 never caused high molecular weight smear formation of p210$^{bcr\ abl}$ in these experiments. Figure 5(B) demonstrates that autophosphorylation of p210$^{bcr\ abl}$ in immune complexes is completely suppressed by AG957 but not by AG555. Thus, under conditions where AG957 is effective as a tyrosine kinase antagonist (Figure 5B), it induces abnormal size of its putative target (Figures 5A and C), analogous to the effect of AG957 on p210$^{bcr\ abl}$ in living cells. Figure 5(D) demonstrates that DTT can suppress the capacity of 50 μM AG957 to induce > 210 kDa smear formation in immunoprecipitates formed with anti-p210$^{bcr\ abl}$ from $^{32}PO_4$-labeled cells.

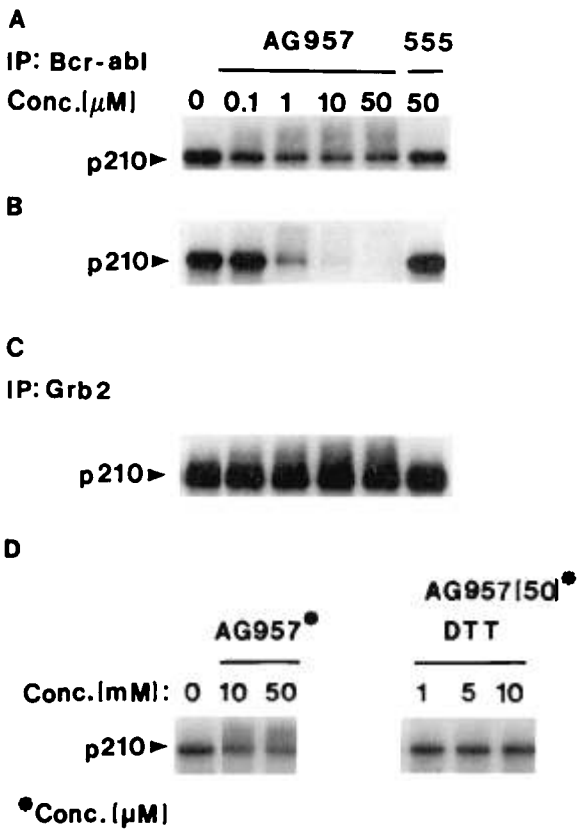

Figure 5. Effect of *in vitro* AG957 on p210$^{bcr\ abl}$. Anti-p210$^{bcr\ abl}$ immune complexes were prepared from K562 cells metabolically-labeled with 1 mCi/ml [^{32}P]orthophosphate for 4 h (10^7 cells 1 ml) (A and C) or from unlabeled cells (B). Complexes were suspended in PLC buffer and exposed (A and C) to AG957 or AG555 at the indicated concentration for 30 min at 30°C, or alternatively, received [γ-^{32}P]ATP (B) in the presence of the indicated concentration of AG957 or AG555. In (D), a reaction using anti-Bcr immunoprecipitate from labeled cells was conducted with increasing concentration of AG957 (left lanes) or with 50 μM AG957 in the presence of the indicated concentration of DTT. Reactions were stopped by addition of 10 μl of 4 × SDS sample buffer. Samples were heated at 100°C for 5 min and proteins were resolved on 4 20% gradient gels.

Discussion

The experiments presented here demonstrate that the tyrphostin AG957 can alter the physical state of p210$^{bcr\ abl}$ and the association of p210$^{bcr\ abl}$ with Shc and Grb2 in K562 cells. Specifically, AG957 causes immunoreactive p210$^{bcr\ abl}$ to migrate at > 210 kDa, as revealed in direct immunoprecipitates and as suggested by material detected by Western blotting with anti-phosphotyrosine antibodies but immunoprecipitated with anti-Shc or anti-Grb2. The abnormally migrating p210$^{bcr\ abl}$ is observed also in cells labeled with [^{32}P]orthophosphate or [^{35}S]methionine. The amount of native p210$^{bcr-abl}$ in immunoprecipitates exposed to AG957 *in vitro* declines with the appearance of material migrating with an apparently higher molecular weight. AG957 also decreases the tyrosine phosphorylation of native Shc.

The most likely explanation for these effects in K562 cells is that AG957 is acting to covalently modify the p210$^{bcr\ abl}$ signaling complex. This could result in the cross-linking of p210$^{bcr\ abl}$ to itself or to signaling molecules such as Shc. p210$^{bcr\ abl}$ is known to form intracellular associations with itself in the form of tetramers,[30] the actin cytoskeleton,[31] syp,[15] fes[33] and the 14-3-3 family of proteins.[34] Interestingly, Grb2, which clearly associates with p210$^{bcr\ abl}$ through tyrosine 177, does not appear with altered molecular weight after exposure to AG957. Thus, not all p210$^{bcr\ abl}$-associated molecules are affected. Indeed, the appearance of total cellular proteins in extracts from cells labeled with either [^{32}P]orthophosphate or [^{35}S]methionine and exposed to AG957 is no different from vehicle-treated cells.

A potential mechanism for AG957 to act as a covalent modifier of target proteins might involve oxidation of AG957 to yield a quinone, which then could act as a nucleophile, for sulfhydryl or amine groups. Reoxidation of the resulting semiquinone could then allow reaction with a second sulfhydryl or an amine functionality.[35] If these were to originate from different proteins in the multimolecular $p210^{bcr\ abl}$ signaling complex, then a mechanism for generating a higher molecular weight form of $p210^{bcr\ abl}$ is apparent, as well as for the disappearance of native $p210^{bcr\ abl}$ and native Shc. This possible mechanism is further supported by our data in that preincubation of immunoprecipitates with 1 mM of DTT prior to the addition of AG957 suppresses the appearance of high molecular weight form of protein of $p210^{bcr\ abl}$ which can be induced to form *in vitro* from prelabeled $p210^{bcr\ abl}$ (Figure 5D). A precedent for this type of mechanism has been shown in the cross-linking of ribonuclease by benzoquinone.[36] While this paper was in preparation, Stanwell *et al.*[37] presented evidence that the structurally related erbstatin analog methyl-2,5-dihydrocinnamate could cross-link many proteins in normal and neoplasic cells, and proposed that this mechanism of cytotoxicity was independent of tyrosine kinase inhibition. In the case of AG957, however, our experiments demonstrate production of an abnormal form of $p210^{bcr\ abl}$ at a 4-fold lower concentration of drug (25 μM), under conditions where most cellular proteins are not affected in appearance by metabolic labeling. This raises the possibility that AG957 may have relative selectivity for $p210^{bcr\ abl}$, concordant with the prior demonstrations[24,25] of the moderately potent capacity of the drug to inhibit $p210^{bcr\ abl}$ tyrosine kinase. The relationship of AG957's ability to inhibit the tyrosine kinase activity of $p210^{bcr\ abl}$ to its capacity to alter its physical state remains to be clarified.

Veillette *et al.*[38] have previously noted that cysteines 464 and 475 in $p56^{lck}$ are critical for catalytic function or protein half-life. Of interest, these cysteines are highly conserved throughout the tyrosine kinase family of signaling molecules, including *abl*.[38] Thus, nucleophiles reactive with critical cysteine residues in tyrosine kinase active sites or substrate recognition sites might be expected to cause structural modifications of such proteins with inhibition of catalytic activity. Selectivity for a particular kinase could, therefore, emerge from two potential influences. The intrinsic affinity of the drug for the kinase would confer one element of selectivity and the rate or capability to react with nucleophilic groups would be a second basis for inhibition. In our case, AG957 appears to selectively alter $p210^{bcr\ abl}$, as compared to the lack of apparent interaction of the drug with the vast majority of cellular proteins and phosphoproteins (Figures 3A and 4A).

The experiments presented here offer a different perspective from which to consider the development of the tyrphostin class of tyrosine kinase antagonist. While these agents in purified enzyme preparations under some circumstances apparently can act as kinetically reversible antagonists,[24] our experiments clearly show (Figure 5) that under conditions where AG957 can efficiently inhibit $p210^{bcr\ abl}$ tyrosine kinase activity in cells and in immune complexes, it is altering the physical state of the enzyme (Figures 1–4) through a mechanism that is sensitive to thiols (Figure 5). Thus, tyrphostins such as AG957 might be thought of as target-selective or target-restricted alkylating agents, which if structurally optimized, might prove effective in irreversibly inhibiting a tyrosine kinase pathway by altering the target's physical state or normal associations with downstream signaling molecules. From this point of view, it may conceivably be desirable to enhance the capacity of AG957 to act as a covalent modifier of target signaling molecules, particularly if these alterations result in a more potent growth inhibitor. Successive generations of $p210^{bcr\ abl}$-directed molecules and perhaps drugs directed at other tyrosine kinase targets could acquire enhanced potency and therapeutic index by specifically and irreversibly altering target tyrosine kinases by such mechanisms. A basis for the improvement of the therapeutic index in this class of growth inhibitors could be the design of molecules which capitalize on the irreversible modification of the signaling pathways dominant in a particular cell type, while seeking to spare other signaling systems in normal cell types.

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