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Effects of Tyrphostins on the Activated c-*src* Protein in NIH/3T3 Cells

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SUMMARY

Tyrphostins are synthetic compounds that have been described as *in vitro* and *in vivo* inhibitors of epidermal growth factor receptor tyrosine kinase activity. In NIH/3T3 cells transfected with the c-*src*/F527 gene, an increase in the level of tyrosine phosphorylation of several proteins, including pp125^{FAK}, within a group of proteins of 120 kDa, of p85 (cortactin), and of p62 is observed, which is due to the elevated kinase activity of the resulting encoded pp60^{F527} protein. In the transfected cells, we showed that the tyrphostins we used, i.e., AG18, AG34, and AG82, strongly diminished the tyrosine phosphorylation of these proteins. Analysis of the steady state level of pp60^{F527} in tyrphostin-treated cells revealed that AG34 and AG82, the two most potent compounds, also induced 30 and 48% decreases, respectively, in the amount of pp60^{F527}, while having no action on the levels of other proteins, especially the pp60^{F527} kinase substrates. Measurement of the rates of pp60^{F527} synthesis and degradation showed that this decreased level was due to a slower rate of synthesis in the presence of AG34 and AG82. Tyrphostins also reversed the pp60^{F527}-induced transformed morphology of NIH/3T3 cells and also inhibited the pp60^{F527} kinase activity *in vitro*. We conclude that the effects elicited by the tyrphostins occurred not only through the inhibition of the pp60^{F527} protein kinase activity but also through a selective reduction of the Src protein steady state level in the cases of AG34 and AG82. This is a novel mode of action for these two tyrphostins, which were the most active compounds in this system.

The transforming Rous sarcoma virus-encoded pp60$^{v\text{-}src}$, and its normal cellular counterpart pp60$^{c\text{-}src}$, are members of a family of protein tyrosine kinases involved in the regulation of cell growth and differentiation. The kinase activity of pp60$^{c\text{-}src}$ and of other members of this family is negatively regulated by phosphorylation at a carboxyl-terminal tyrosine residue, Tyr-527 in pp60$^{c\text{-}src}$ (1), making pp60$^{c\text{-}src}$ nontransforming even when overexpressed (2).

Substitution of Tyr-527 by phenylalanine, which cannot be phosphorylated, removal of Tyr-527 by truncation of the pp60$^{c\text{-}src}$ carboxyl terminus, or formation of a complex with the middle T antigen of polyoma virus stimulated the protein tyrosine kinase activity and the transforming potential of pp60$^{c\text{-}src}$ variants (3). Expression of pp60$^{v\text{-}src}$ or activated forms of pp60$^{c\text{-}src}$, i.e., pp60^{F527}, resulted in the increased tyrosine phosphorylation of several proteins, among which pp125^{FAK}, GAP, p85, and p62 have already been characterized (4–8).

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Protein tyrosine kinase inhibitors acting on ligand-activated growth factor receptors and on a number of oncogene products have been described (9, 10). These drugs behave as competitive ligands towards either the ATP or peptide binding sites. Among them, the most extensively studied are genistein, erbstatin, and tyrphostins. Genistein, an ATP binding site competitor, inhibits EGF-R tyrosine kinase activity both *in vitro* and *in vivo* (11). However, it also behaves as a S6 kinase (12) and a DNA topoisomerase II inhibitor (13). Erbstatin (14) competes with the peptide binding site. It inhibits EGF-R tyrosine kinase activity both *in vitro* (15) and in intact cells (16) and also inhibits pp60$^{v\text{-}src}$ tyrosine kinase activity when added to Rous sarcoma virus-infected cells (15). Tyrphostins are synthetic compounds previously described with respect to their inhibitory activity on the EGF-dependent proliferation of keratinocytes, A431 clone 15 cells, and HER14 cells, and their antiproliferative actions are correlated with inhibition of EGF-R tyrosine kinase activity in intact cells and also *in vitro* (17–19). Tyrphostins also block EGF-induced phospholipase Cγ phosphorylation, Ca^{2+} release in living cells, and phosphoinositide production (20).

ABBREVIATIONS: GAP, GTPase-activating protein; EGF-R, epidermal growth factor receptor; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; Me_2SO, dimethylsulfoxide; PTyr, phosphotyrosine; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAb, monoclonal antibody.

Among all of the tyrphostins, some derivatives have been described as *in vitro* EGF-R and p210$^{bcr\text{-}abl}$ tyrosine kinase activity inhibitors (17, 21, 22). In the present study, we investigated the effects of three tyrphostins, AG18, AG34, and AG82 (Fig. 1), on the pp60^{F527} tyrosine kinase activity in intact cells and *in vitro*. We found that the tyrphostins inhibited this kinase activity *in vitro* and in intact cells and reversed the transformed morphology of pp60^{F527}-expressing cells; two of the tyrphostins could selectively reduce the steady state level of pp60^{F527}, pointing to a novel mechanism of action of these molecules.

Materials and Methods

Antibodies. The mAb 327 (Oncogene Science, Inc.) recognized the c-*src* protein. The anti-p85 (mAb 4F11) and anti-pp125^{FAK} (mAb 2A7) mAbs were kindly provided by Dr. J. T. Parsons University of Virginia, Charlottesville, Virginia. Anti-PTyr mAb and anti-GAP polyclonal antibodies were from Upstate Biotechnology, Inc.

Cells and plasmids. NIH/3T3 cells, ψ2 cells, and pZIP-Neo SV(X) shuttle vector were from Dr. F. Dautry, Institute Gustave Roussy, Villejuif, France; the plasmid pGEM4-F527, containing the chicken c-*src* mutant, was a gift from Dr. W. Eckhart, The Salk Institute, La Jolla, California. The cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics.

A 1.6-kb *Bam*HI-*Hind*III fragment containing the c-*src* mutant cDNA was isolated from the plasmid pGEM4-F527, blunt-ended, and inserted into the blunt-ended *Bam*HI site of the retrovirus expression vector pZIP-Neo SV(X). This generated the pZIP-Neo/F527 recombinant retroviral vector.

Generation of helper-free recombinant retroviruses and cell lines expressing pp60^{F527} protein. The recombinant retroviral vector pZIP-Neo/F527 and the parental retroviral vector pZIP-Neo SV(X) were transfected into ψ2 packaging cells, and stable virus producer lines were obtained with a viral titer of 1×10^4 colony-forming units/ml. Recombinant retroviruses were used to introduce the gene encoding for pp60^{F527} into NIH/3T3 cells, and parental retroviruses were used to produce control NIH/3T3 cells. Briefly, to infect the NIH/3T3 cells, 1 ml of the transfected ψ2 cell supernatant was added to 1×10^6 NIH/3T3 cells in a 100-mm-diameter dish and incubated for 4 hr in the presence of 8 μg/ml polybrene (Aldrich). Complete medium was then added, cultures were incubated for 2 days, and G418 (650 μg/ml) selection was imposed. The resistant NIH/3T3 colonies were screened for the presence of pp60^{F527} protein using mAb 327 (Oncogene Science).

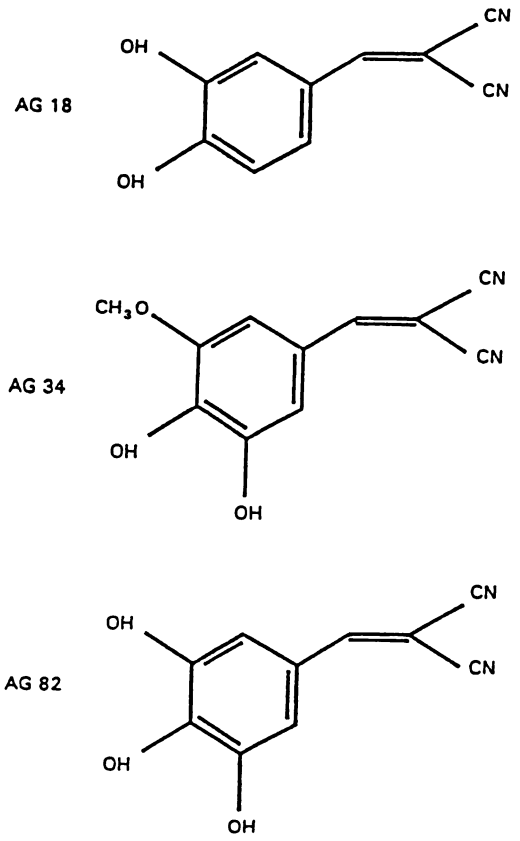

Fig. 1. Structures of the different tyrphostins.

Cell culture. The tyrphostins were synthesized as described by Gazit *et al.* (23), solubilized, and diluted in Me_2SO before use. Drug concentrations were adjusted to have a final Me_2SO concentration of 1% in the cell growth medium. The same Me_2SO concentration was added to the nontreated cells. This Me_2SO concentration had no effect on the cell proliferation or on the level of protein tyrosine phosphorylation.

To study the effect of tyrphostins on tyrosine phosphorylation of proteins in the different NIH/3T3 cell lines, $7\text{–}8 \times 10^5$ cells were plated in 100-mm dishes for 24 hr, after which the medium was replaced by fresh medium containing different concentrations of the drugs. Cells were grown for an additional time, ranging from 1 to 24 hr, in the presence of the drugs and were processed as described for immunoblotting (see below).

For morphological studies, 1.2×10^5 cells were seeded in a 25-cm^2 flask for 24 hr, after which the medium was replaced with medium containing increasing concentrations of tyrphostins ranging from 5 to 100 μM. Cells were exposed to drugs for 72 hr and photographs were taken using a microscope (IMT-2; Olympus).

Western blotting and immunoprecipitation. Exponentially growing cells (1.5×10^6) were disrupted at 4° in 200 μl of buffer A (1% Nonidet P-40, 50 mM Tris·HCl, pH 7.6, 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin). Lysates were clarified by centrifugation at $15{,}000 \times g$ for 15 min at 4°. Protein concentrations were determined using bicinchoninic acid reagent (Pierce).

Protein extracts (100 μg) were directly fractionated by 8% SDS-PAGE. After electrophoretic transfer to nitrocellulose (BA-S 85; Schleicher and Schuell), the filters were incubated for 1 hr at 4° with mAb 327 (1 μg/ml) (Oncogene Science, Inc.) or overnight with mAb 4F11 (5 μg/ml), mAb 2A7 (1/250), anti-PTyr mAb (1/2000) (Upstate Biotechnology), or polyclonal anti-GAP antibodies (1/2500) (Upstate Biotechnology). The proteins recognized by these antibodies were visualized by incubation of the filters with a second antibody, conjugated with horseradish peroxidase. The filters were developed using the enhanced chemiluminescence procedure (Amersham).

For immunoprecipitation, 250 μg of cellular extracts in 250 μl of buffer A were incubated at 4° for 2–4 hr with 0.5 μg of mAb 327, 2.5 μg of mAb 4F11 or mAb 2A7 (2 μl), anti-GAP antibodies (1/25), or 2 μg of anti-PTyr mAb. Immunocomplexes were recovered by the addition of 10% (v/v) Protein A-Sepharose beads (Sigma) for mAb 327, anti-PTyr antibodies, and anti-GAP antibodies or 10% (v/v) Protein A-Sepharose beads that had been preincubated with 5 μg of rabbit anti-mouse IgG (Nordic) for mAb 4F11 and mAb 2A7. The beads were washed three times in buffer A, proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose, and immunoblotting was performed as described previously.

[^{35}S]Methionine labeling of cells. To determine the synthesis rate of pp60^{F527}, cells were seeded at 3×10^5/35-mm dish and were allowed to grow for 24 hr. The cells were then incubated in 2 ml of methionine-free DMEM containing 30 μg/ml L-methionine and supplemented with 4% dialyzed calf serum, with or without 100 μM tyrphostins, for 24 hr before labeling. [^{35}S]Methionine (Tran[^{35}S] Label; >1000 Ci/mmol as purchased from ICN Radiochemicals, Orsay, France) was added at 100 μCi/ml and the culture was incubated for up to 2 hr, always in the presence or not of tyrphostins.

To determine the degradation rate of pp60^{F527}, cells were seeded at 3

× 10^5/35-mm dish, allowed to grow for 24 hr, and then incubated in 1 ml of methionine-free DMEM, supplemented with 4% dialyzed calf serum, for 1 hr before labeling. The cells were then incubated for 2 hr in 1 ml of the same medium containing 50 μCi of [^{35}S]methionine (pulse labeling). The labeled cells were then either lysed immediately or further incubated for up to 24 hr (chase) with 2 ml of growth medium containing 150 μg/ml unlabeled methionine (Sigma). Tyrphostins were added or not added during this chase period.

In both cases, cells were lysed in radioimmunoprecipitation assay buffer (20 mM Tris·HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM PMSF) and the whole-cell lysates were subjected to pp60^{F527} immunoprecipitation using mAb 327, as described above.

Construction of a plasmid expressing the pp60^{F527} mRNA sequence, pGEM-3Z/seq5′-F527. A *Sac*I-*Sac*I fragment (a 2300-base pair portion containing the coding sequence of *src* and the 5′ untranslated region) was isolated from the recombinant pZIP-Neo/F527 vector. This fragment was then cloned under the T7 promoter of the pGEM-3Z vector (Promega) at the *Sac*I site, giving the plasmid pGEM-3Z/seq5′-F527. Transcription and concentration determination were then carried out according to conventional methods. The size of the mRNA synthesized was checked on an agarose gel.

***In vitro* translation.** The *in vitro* translation inhibition by the tyrphostins was assayed in 25 μl of a rabbit reticulocyte lysate (Promega) programmed with three different mRNAs, i.e., a commercial luciferase mRNA (1.9 kb) (Promega), a strict F527 mRNA (1.6 kb) synthesized using the pGEM4-F527 plasmid (see above), and an mRNA containing both the 5′ untranslated region and the coding region of pp60^{F527}, using the pGEM-3Z/seq5′-F527 construct (2.3 kb). The incubations were carried out at 30° for 2 hr with Tran[^{35}S] Label (ICN, Orsay, France) in the presence or absence of 100 μM tyrphostins (in 1% Me_2SO, final concentration), the samples were then denatured, and the proteins were separated by 8% SDS-PAGE. After fluorography, the bands corresponding to pp60^{F527} were quantified with a Joyce Loebl chromoscan densitometer. Each experiment was performed twice.

Immune complex kinase assays. For the *in vitro* studies, pp60^{F527} was immunoprecipitated with mAb 327, from 250 μg of cellular extracts prepared as for the Western blotting experiments. The immune complex was rinsed three times in buffer B (25 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM dithiotreitol, 10%, v/v, glycerol, 1 mM PMSF, 0.1% Triton X-100, 10 μg/ml aprotinin). Beads were resuspended in 50 μl of kinase reaction buffer (20 mM HEPES-KOH, pH 7.0, 6 mM $MgCl_2$, 20 mM sodium orthovanadate). Then, 1 μg of acid-denatured enolase was added, the tyrphostins were added, and the reaction was immediately initiated by addition of 3.5 μM ATP containing 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). No preincubation of the tyrphostins with pp60^{F527} was required. Incubations were carried out for 10 min at 30°. The reaction was stopped by addition of 17 μl of 3× sample buffer (1× buffer was composed of 125 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol). The samples were then boiled for 5 min at 100° and analyzed by 8% SDS-PAGE, followed by autoradiography.

Results

Evidence that expression of pp60^{F527} increases substrate PTyr contents within cells. Recombinant retroviruses were used to introduce the c-*src*/F527 gene into NIH/3T3 cells, as described in Materials and Methods. The control cells were NIH/3T3 cells infected with the vector pZIP-Neo SV(X). Expression of pp60^{F527} in NIH/3T3 cells led to an increase in tyrosine phosphorylation of several proteins, compared with the NIH/3T3 control cells, i.e., 3-fold and 12-fold increases for a group of 120-kDa proteins (p120) and for an 85-kDa protein (p85), respectively (Fig. 2a, *lane 1*, compared with *lane 5*). In addition, a 62-kDa protein (p62) appeared to be tyrosine phosphorylated only in cells expressing pp60^{F527} (Fig. 2a, *lane 1*, compared with *lane 5*).

Reduction of protein tyrosine phosphorylation by tyrphostins in intact cells. Tyrphostins were first characterized as being inhibitors of the EGF-R tyrosine kinase activity in many cell types (17–19). In an attempt to better define this tyrosine kinase inhibition, we investigated whether they could alter the increase in cellular PTyr content attributable to the elevated tyrosine kinase activity of pp60^{F527}. Lyall *et al.* (19) reported that relatively high concentrations of the tyrphostin AG18 (>25 μM) and prolonged incubation periods (16–24 hr) were required to modulate EGF-R activities in intact HER14 cells.

Western blotting analysis, with anti-PTyr antibodies, of the tyrosine-phosphorylated proteins in pp60^{F527}-expressing cells treated with 50 or 100 μM tyrphostins (Fig. 1) for 1 or 6 hr did not show any reduction of PTyr content (data not shown). However, cells treated for 24 hr with 100 μM tyrphostins showed a decrease in the tyrosine phosphorylation of cellular proteins, compared with the nontreated cells (Fig. 2, a, *lanes 6-8*, compared with *lane 5*, and b). However, the degree of reduction was different for each protein and for each drug. AG18-treated cells showed a reduction in tyrosine phosphorylation of the p120, p85, and p62 proteins of 45%, 65%, and 55%, respectively (Fig. 2, a, *lane 6*, and b). Treatment with AG34 or AG82 also reduced the level of tyrosine phosphorylation; the decrease was 60% for p120, 80% for p85, and 75% for p62 (Fig. 2, a, *lanes 7* and *8*, and b). The experiment shown in Fig. 2 is representative of four independent experiments exhibiting the same pattern of reduction. Treatment of control cells with the tyrphostins under the same conditions did not modify the basal level of substrate tyrosine phosphorylation (Fig. 2a, *lanes 2-4*, compared with *lane 1*).

To further confirm the reduction of tyrosine phosphorylation by tyrphostins in intact cells, the treated control cells and the treated pp60^{F527}-expressing cells were immunoprecipitated with anti-PTyr antibodies, electrophoresed, transferred to nitrocellulose, and immunoblotted with the same anti-PTyr antibodies. As in the whole-cell lysate experiment, the immunoprecipitation experiment clearly showed that proteins from control cells were slightly phosphorylated, compared with proteins from nontreated pp60^{F527}-expressing cells (Fig. 2c, *lanes 1-4*, compared with *lane 5*). In addition, the treatment by the tyrphostins did not alter this basal level of phosphorylation. In the tyrphostin-treated pp60^{F527}-expressing cells, AG34 and AG82 were the most potent tyrosine phosphorylation-reducing compounds. The extents of phosphorylation reduction with these three tyrphostins in the anti-PTyr immunoprecipitation experiment were quite similar to those in the whole-cell lysate experiment.

These results demonstrated that the tyrphostins reduced the level of tyrosine phosphorylation of the different pp60^{F527} substrates. This reduction could be due to an inhibitory effect of the tyrphostins on the pp60^{F527} kinase activity or to a reduction of the substrate protein levels. The next step of our investigations was to evaluate the levels of pp60^{F527} and its substrates under the experimental conditions, to discriminate between these two possibilities.

Levels of the substrate proteins and of their phosphorylation in tyrphostin-treated cells. Using antibodies directed against pp60^{src} (mAb 327), p85 (mAb 4F11), GAP (anti-GAP antibodies), and pp125^{FAK} (mAb 2A7), we performed

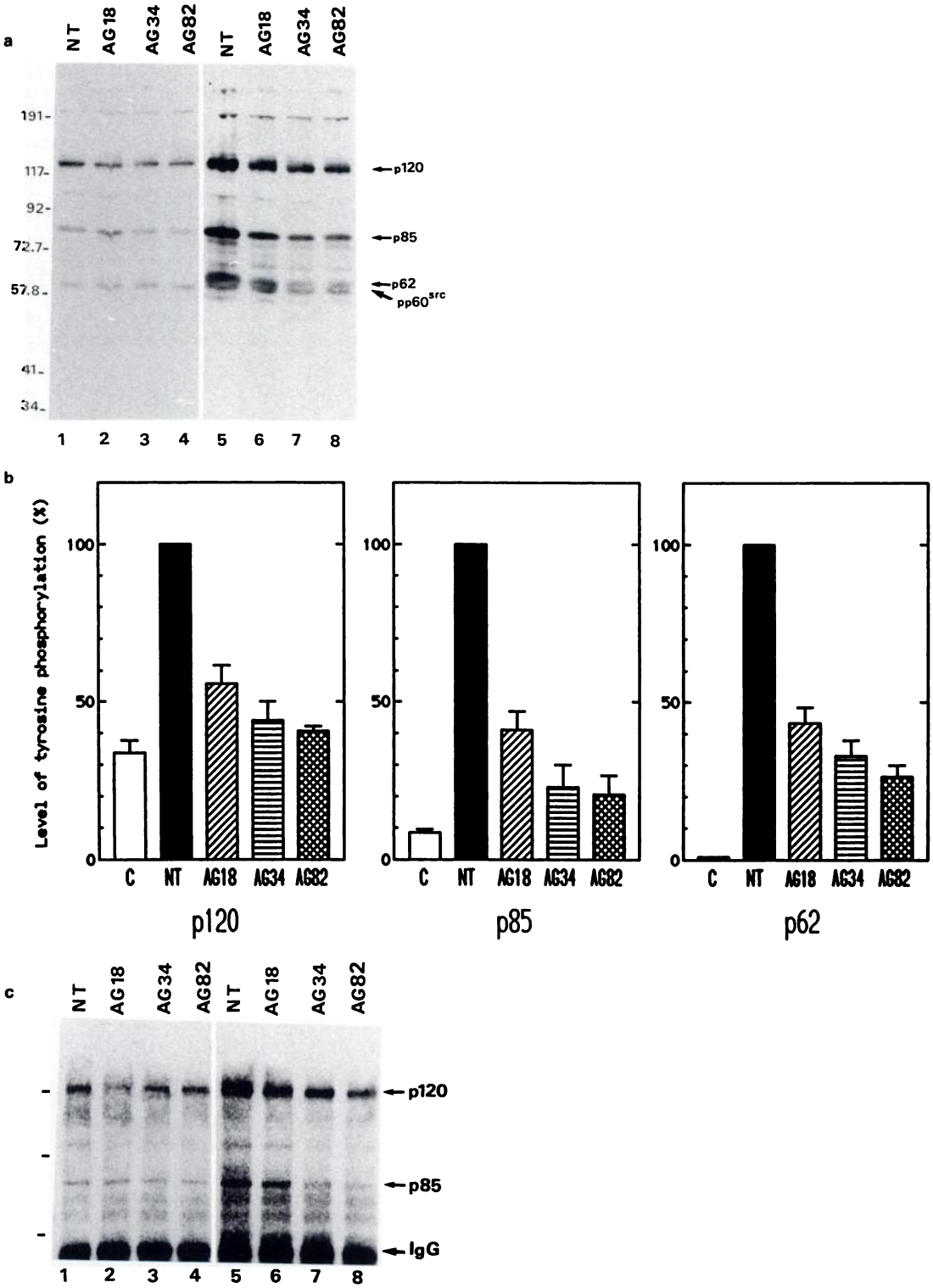

Fig. 2. Effect of the tyrphostins on the cellular substrate PTyr content of pp60^{F527}-expressing NIH/3T3 cells. a, Level of substrate PTyr content in tyrphostin-treated cells. Cells were treated with 100 μM tyrphostins for 24 hr and lysed, and proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-PTyr mAb. *Lanes 1-4*, *neo*-transfected control cells; *lanes 5-8*, pp60^{F527}-expressing cells. *Lanes 1* and *5*, nontreated cells; *lanes 2* and *6*, AG18-treated cells; *lanes 3* and *7*, AG34-treated cells; *lanes 4* and *8*, AG82-treated cells. This Western blot is representative of three independent experiments. *Numbers on the left*, positions of the molecular mass markers (in kDa). b, Quantitation by densitometric scanning of the tyrphostin-induced reduction of cellular substrate PTyr content analyzed by Western blotting. *C*, nontreated control cells; *NT*, nontreated pp60^{F527}-expressing cells. The values represent the average of three independent experiments. The 100% value always represents the PTyr content in nontreated pp60^{F527}-expressing cells. c, Evidence that tyrphostins inhibit tyrosine phosphorylation by pp60^{F527} in intact cells. Nontreated and tyrphostin-treated cells were immunoprecipitated with anti-PTyr antibodies. Immunoprecipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-PTyr antibodies. Lanes are as in a. *Dashes*, positions of the molecular mass markers.

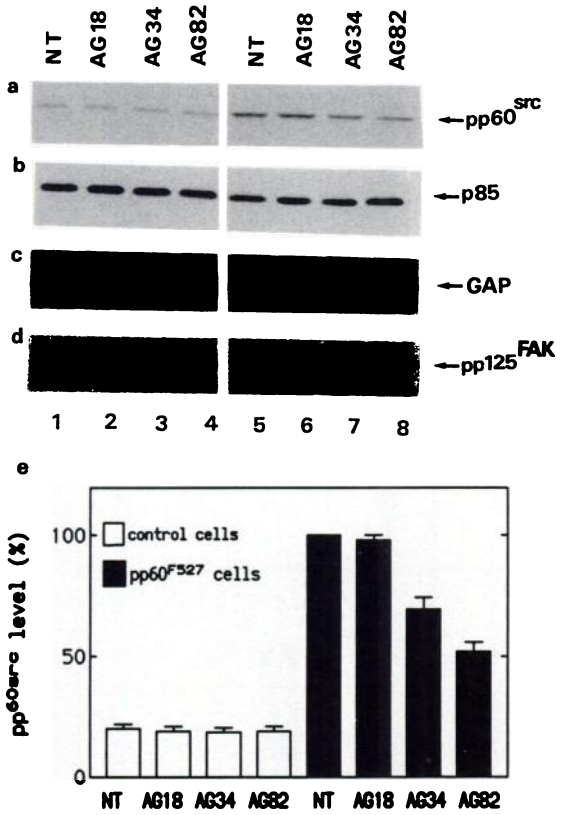

Fig. 3. Effect of the tyrphostins on protein steady state levels in treated cells. Whole-cell lysates of control cells (*lanes 1-4*) and pp60^{F527}-expressing cells (*lanes 5-8*) were separated by SDS-PAGE, transferred to nitrocellulose, and individually immunoblotted with anti-src mAb 327 (a), anti-p85 mAb 4F11 (b), anti-GAP antibodies (c), and anti-pp125^{FAK} mAb 2A7 (d). The data shown here are representative of four independent experiments. e, Quantitation of the pp60^{src} level by densitometric scanning of the anti-src immunoblot. *NT*, nontreated cells. The values represent the average of four independent experiments.

individual immunoblots on whole-cell lysates to evaluate the amount of each protein in each case. The results in Fig. 3, a and e, showed that AG34 and AG82 induced decreases in the pp60^{F527} steady state level of approximately 30 and 48%, respectively, compared with the nontreated cells. The same effect of AG34 and AG82 on pp60^{F527} levels was consistently observed in each of the four independent experiments. Clearly, the decrease of src protein levels induced by AG34 and AG82 (30 and 48%, respectively) was not sufficient to account completely for the reduction of the substrate phosphorylation levels (65–85%). This effect was not observed either on the endogenous pp60^{src} levels in control AG34- and AG82-treated cells (Fig. 3, a, *lanes 3* and *4*) or on the substrate protein levels, i.e., p85, GAP, and pp125^{FAK}, in either cell line (Fig. 3, b-d, *lanes 3*, *4*, *7*, and *8*). AG18, although it decreased the PTyr content in treated cells (60%), had no effect on either the src protein or substrate protein levels in pp60^{F527}-expressing cells or in control cells (Fig. 3, a-d, *lanes 2*, *5*, *7*, and *10*). These results suggested that the decrease of pp60^{F527} steady state levels induced by AG34 and AG82 was specific for this protein.

To individually investigate the phosphorylation state of each protein in the tyrphostin series, lysates of treated cells were immunoprecipitated with the specific antisubstrate antibodies (mAb 4F11, mAb 2A7, and anti-GAP antibodies). The immunoprecipitates were then electrophoresed, transferred, and immunoblotted with the same antisubstrate antibodies, to first quantitate protein levels. The treatment of pp60^{F527}-expressing cells with tyrphostins did not alter p85, pp125^{FAK}, or GAP protein levels, which were identical to those in nontreated control cells (Fig. 4, a, b, *left*, and c). The same kind of experiment performed with anti-src antibodies exhibited two features; (i) in control nontransfected cells a low level of the src protein was observed that, as expected, was increased in nontreated transfected cells (Fig. 4d, *lanes 1* and *2*) and (ii) in the tyrphostin-treated cells AG18 did not affect the steady state level of pp60^{F527} but AG34 and AG82 did, reinforcing the results of the whole-cell lysate experiment (Fig. 4d).

The same immunoprecipitates were electrophoresed and immunoblotted with anti-PTyr antibodies to determine the phosphorylation level of the immunoprecitated proteins. The increase in PTyr content of p85 and pp125^{FAK} in pp60^{F527}-expressing cells, compared with control cells, was clearly detected (Fig. 4, a and b, *right*, *lanes 6*, compared with *lanes 7*). In addition, the inhibition of phosphorylation in the immunoprecipitates of tyrphostin-treated lysates was clearly observed for the p85 protein (Fig. 4, a, *right*, *lane 7*, compared with *lanes 8-10*). The anti-GAP immunoprecipitates immunoblotted with anti-PTyr antibodies did not show any detectable level of GAP phosphorylation (data not shown). This result was consistent with previous reports showing that GAP was indeed only slightly phosphorylated in v-*src*-transformed CEF cells.

Taken together, these data clearly demonstrated that p85 and pp125^{FAK}, two already identified and characterized proteins (5, 7, 8), are tyrosine phosphorylated by pp60^{F527}. However, only the phosphorylation of p85 was inhibited by the tyrphostins. The most potent inhibitors were AG34 and AG82. In the case of pp125^{FAK}, immunoblotting with anti-PTyr antibodies

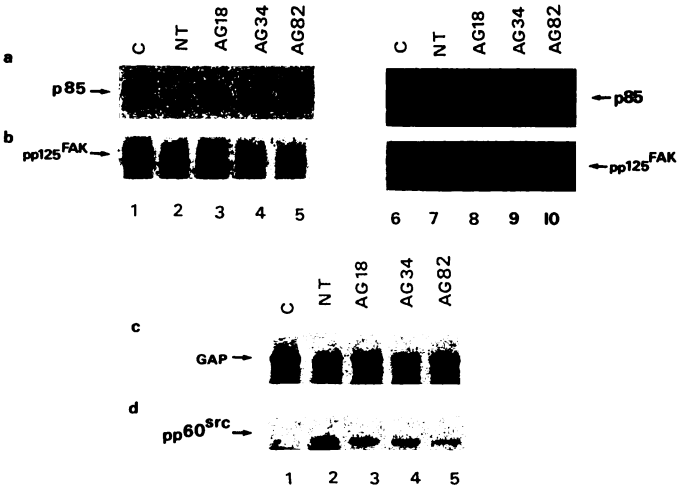

Fig. 4. Tyrosine phosphorylation of p62, p85, and pp125^{FAK} in intact pp60^{F527}-expressing cells and inhibition of this phosphorylation by the tyrphostins. pp60^{F527}-expressing cells treated with 100 μM tyrphostins for 24 hr were lysed, and proteins (250 μg) were immunoprecipitated with anti-p85 mAb 4F11 (a), anti-pp125^{FAK} mAb 2A7 (b), anti-GAP antibodies (c), or anti-src mAb 327 (d). Immunoprecipitates were separated by 8% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-p85 mAb 4F11 (a, *left*), anti-pp125^{FAK} mAb 2A7 (b, *left*), anti-GAP antibodies (c), or anti-src mAb 327 (d). The same immunoprecipitates, electrophoresed and transferred to nitrocellulose, were also immunoblotted with anti-PTyr antibodies (a and b, *right*). *Lanes 1* and *6*, nontreated control cells (*C*); *lanes 2* and *7*, nontreated pp60^{F527}-expressing cells (*NT*); *lanes 3* and *8*, AG18-treated cells; *lanes 4* and *9*, AG34-treated cells; *lanes 5* and *10*, AG82-treated cells.

revealed that, indeed, the phosphorylation of this protein was not inhibited by AG18 and was very slightly inhibited by AG34 and AG82. These two tyrphostins could also selectively diminish the steady state level of pp60^{F527} while having no effect on other proteins, especially substrate protein steady state levels. This reduction in the amount of pp60^{F527} protein in AG34- and AG82-treated cells suggested that these two molecules affected either the transcription of the transfected c-*src*/F527 gene or the turnover of its product. We decided to test these two possibilities.

Expression of the c-*src*/F527 gene and turnover of the pp60^{F527} protein in tyrphostin-treated cells. We first investigated whether the mRNA level of the c-*src*/F527 gene was altered by tyrphostin treatment. No modification of the c-*src*/F527 mRNA was observed in any of the tyrphostin-treated cases (data not shown).

We then examined the effect of the tyrphostins on the metabolic turnover of pp60^{F527}. For this, proteins were labeled with [^{35}S]methionine and then chased for various length of times. Proteins were then extracted and immunoprecipitated with anti-src mAb 327. Fig. 5a shows the degradation pattern of pp60^{F527} in the presence of AG18 (Fig. 5a, *lanes 6-9*), AG34 (Fig. 5a, *lanes 10-13*), and AG82 (Fig. 5a, *lanes 14-17*), compared with the degradation pattern in the absence of tyrphostins (Fig. 5a, *lanes 2-5*). In the tyrphostin-treated cells, a significant amount of labeled pp60^{F527} still remained undegraded after 24 hr, whereas all of the protein was degraded in the nontreated cells within 24 hr (Fig. 5a, *lanes 9*, *13*, and *17*, compared with *lane 5*). However, the level of the remaining pp60^{F527} in AG18-treated cells was lower than the levels in either AG34- or AG82-treated cells. These results suggested that the tyrphostins protected a portion of pp60^{F527} from degradation.

On the other hand, the rates of pp60^{F527} synthesis differed in the presence of the tyrphostins. This rate was very rapid in the nontreated cells, reaching a maximal level within 1 hr and decreasing within 2 hr (Fig. 5b, *lanes 1-4*). In the presence of AG34 and AG82, this rate was slowed, reaching detectable levels only after >1 hr (Fig. 5b, *lanes 9-16*). In the presence of AG18, although the rate of synthesis was slower than in nontreated cells, measurable levels could be achieved within 30 min (Fig. 5b, *lanes 5-8*). Therefore, the reduction of the steady state level of pp60^{F527} in AG34- and AG82-treated cells was certainly due to a slow rate of synthesis of the protein.

Effect of the tyrphostins on *in vitro* translation of pp60^{F527} mRNA. The effect of the tyrphostins on the translation of different mRNAs was measured in a cell-free extract *in vitro*, as described in Materials and Methods. *In vitro* transcription of c-*src*/F527 mRNA from the pGEM4-F527 plasmid gave rise to an mRNA of 1.6 kb, which was used in an *in vitro*

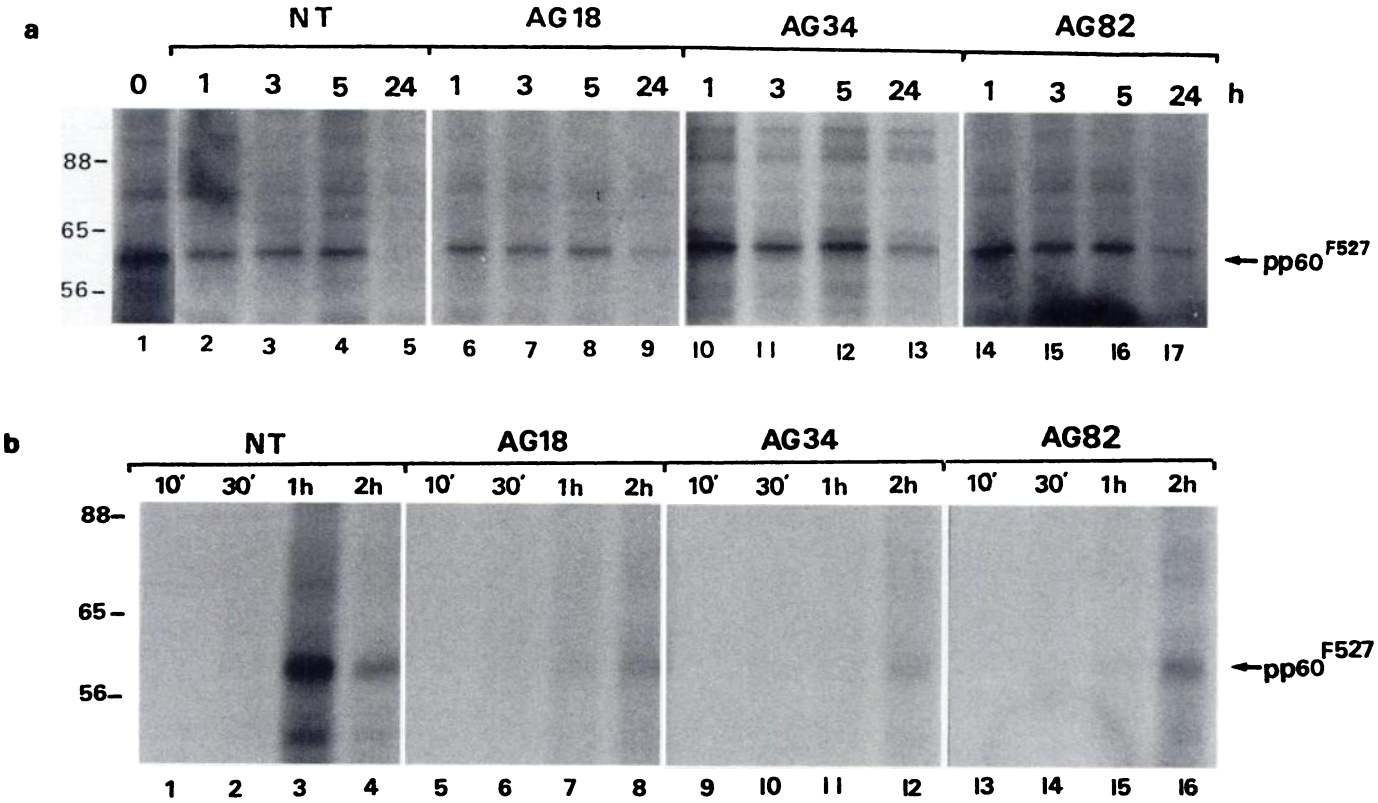

Fig. 5. Effects of tyrphostins on pp60^{F527} at the translational level. a, Degradation of pp60^{F527} in tyrphostin-treated cells. Cells were pulse-labeled for 2 hr and no chase were performed (*lane 1*). Then, 1-, 3-, 5-, and 24-hr chases were performed in the absence of the tyrphostins (*lanes 2-5*) or in the presence of 100 μM AG18 (*lanes 6-9*), 100 μM AG34 (*lanes 10-13*), or 100 μM AG82 (*lanes 14-17*). *Numbers on the left*, positions of the molecular mass markers (in kDa). Cells were lysed in RIPA buffer and clarified, pp60^{F527} was immunoprecipitated with mAb 327, and immunoprecipitates were resolved by SDS-PAGE. The gel was then dried and autoradiographed. These experiments have been performed twice, independently. b, Synthesis rate of pp60^{F527} in tyrphostin-treated cells. Cells were labeled with [^{35}S]methionine for up to 2 hr, after 24-hr pretreatment in the absence of tyrphostins (*lanes 1-4*) or in the presence of 100 μM AG18 (*lanes 5-8*), 100 μM AG34 (*lanes 9-12*), or 100 μM AG82 (*lanes 13-16*), with 10 min (*lanes 1*, *5*, *9*, and *13*), 30 min (*lanes 2*, *6*, *10*, and *14*), 1 hr (*lanes 3*, *7*, *11*, and *15*), or 2 hr (*lanes 4*, *8*, *12*, and *16*) of synthesis. Immunoprecipitations were performed as described for a.

translation assay with rabbit reticulocyte lysate. No modification of the translation of this mRNA was observed when the incubations were carried out in the presence or absence of 100 μM tyrphostins (the translation rates varied from 92 to 98%, compared with control, depending on the tyrphostin tested). The same result was obtained for the translation of an unrelated mRNA (commercial luciferase mRNA).

To mimic more closely the cellular conditions, we constructed the plasmid pGEM-3Z/seq5′-F527, which contained both the sequences 5′ to the *src*/F527 cDNA in pZIP-Neo/F527 and the *src*/F527 cDNA itself (see Materials and Methods). *In vitro* transcription using this plasmid gave rise to seq5′-F527 mRNA (2.3 kb). *In vitro* translation of this mRNA in the presence of the tyrphostins did not result in any modification. Again the translation rate varied between 95 and 100%, depending on the tyrphostin tested.

Effect of tyrphostins on cell morphology. The expression of $pp60^{F527}$ in NIH/3T3 cells induced a transformed morphology, compared with control cells. Transformed cells exhibit a more fusiform shape, are less adherent, and are more refractile than control flat cells (Fig. 6, A, compared with B). To test the potential action of tyrphostins on cell morphology, $pp60^{F527}$-expressing cells were treated with increasing drug concentrations, ranging from 5 to 100 μM, for 72 hr. The lowest concentrations of tyrphostins able to reverse the transformed morphology of the cells were 100 μM for AG18 (Fig. 6C), 50 μM for AG34 (Fig. 6D), and 10 μM for AG82 (Fig. 6E). These results could be corroborated with those observed for the inhibition of tyrosine phosphorylation of p85 and p120; the rank orders observed for morphological reversion and for inhibition of tyrosine phosphorylation were the same.

***In vitro* effects of tyrphostins on the tyrosine kinase activity of $pp60^{F527}$.** The data described above suggested that the tyrphostins could directly inhibit $pp60^{F527}$ tyrosine kinase activity. Hence, we checked whether these molecules could inhibit the src tyrosine kinase activity *in vitro*. The *in vitro* effect of tyrphostins on the tyrosine kinase activity of $pp60^{F527}$ was determined using immune $pp60^{F527}$, as described in Materials and Methods. Several concentrations of the different tyrphostins have been added. Fig. 7 reveals that tyrphostins inhibited $pp60^{F527}$-catalyzed phosphate transfer to the tyrosine residue(s) of the $pp60^{F527}$ protein (autophosphorylation), of enolase (exogenous substrate), and of a 120-kDa protein that coimmunoprecipitated with the src protein (endogenous substrate). IC_{50} values of the tyrphostins were determined for these substrates (Table 1). According to these IC_{50} values, AG34 and AG82 displayed similar inhibitory effects and were again more potent than AG18. However, even at 100 μM, none of the tyrphostins was able to completely inhibit the $pp60^{F527}$ tyrosine kinase activity *in vitro*.

Discussion

Tyrphostins are a series of synthetic compounds that behave as substrate competitive inhibitors of protein tyrosine kinases (17, 21). In several cell types, the antiproliferative action of tyrphostins has been correlated with EGF-R kinase activity inhibition *in situ* (17–19). Some tyrphostins were also found to inhibit Abl protein tyrosine kinase activities *in vitro* (21, 22).

In this work, we have examined whether the tyrphostins could also inhibit the tyrosine kinase activity of $pp60^{F527}$ in intact cells and *in vitro*. The results showed that the tyrphostins also inhibited this activity. For two of them, AG34 and AG82, the inhibitory activity appears to be due to both a decrease in the level of $pp60^{F527}$ protein and a decrease of the catalytic activity of the residual $pp60^{F527}$ kinase. Both molecules were thus apparently more potent than AG18, which decreased only the catalytic activity of the protein.

In intact cells, treatment with 100 μM tyrphostins for 24 hr yielded a significant decrease in cellular protein PTyr content, especially for the p120, p85, and p62 proteins, which are the major $pp60^{F527}$ substrates. AG34 and AG82 were the most effective inhibitors, with a phosphorylation reduction of 80%, 75%, and 60% with p85, p62, and p120, respectively. A decrease in PTyr content was also observed in cells treated with AG18, but it was weaker than the decrease in AG34- and AG82-treated cells. It should be noted that the kinase inhibition was not

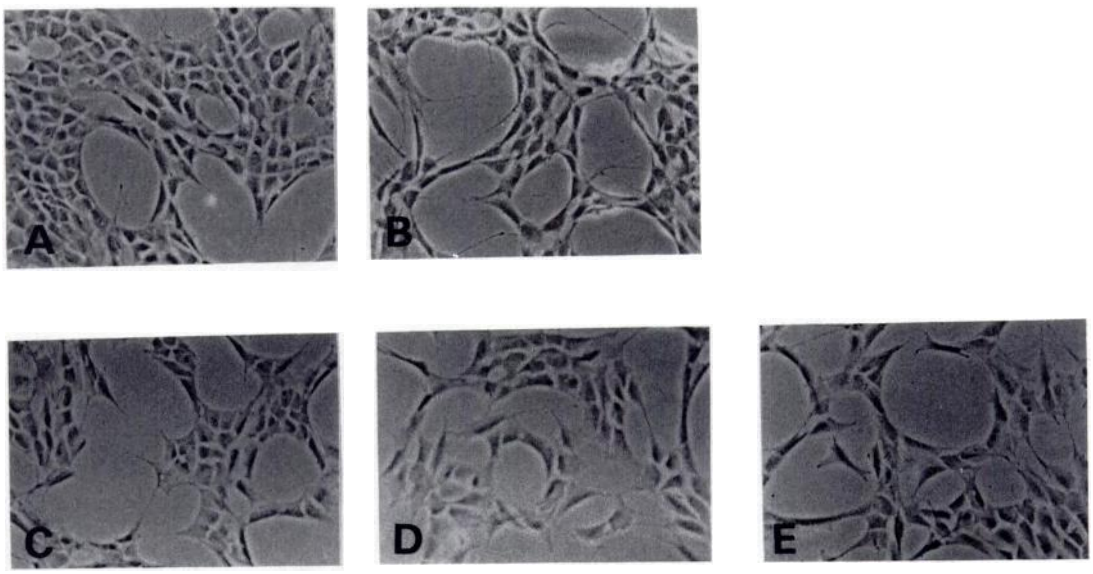

Fig. 6. Light microscopy of $pp60^{F527}$-transformed and tyrphostin-treated cells. A, Control cells; B-E, $pp60^{F527}$-expressing cells. B, Nontreated cells; C, 100 μM AG18-treated cells; D, 50 μM AG34-treated cells; E, 10 μM AG82-treated cells.

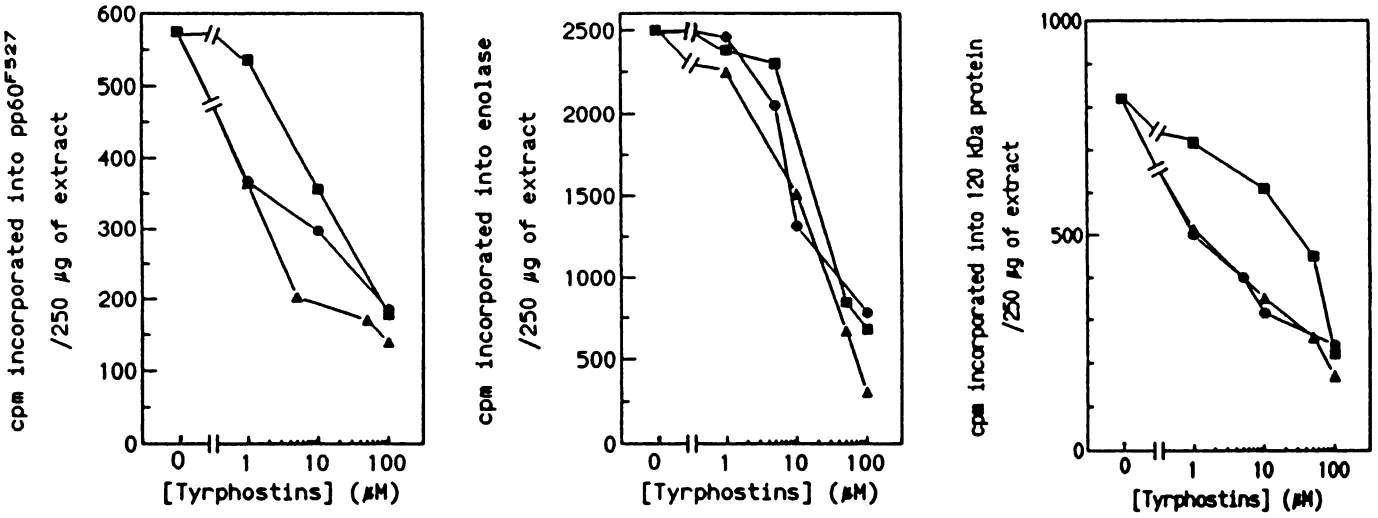

Fig. 7. Inhibitory effects of tyrphostins on *in vitro* protein kinase activity of pp60^{F527}. Immune complex kinase assays were performed *in vitro* with enolase as exogenous substrate. In each case, 0, 1, 5, 10, 50, or 100 µM AG18 (■), AG34 (▲), or AG82 (●) was added. After the reaction (see Materials and Methods), proteins were fractionated by 8% SDS-PAGE. The gel was then stained, dried, and subjected to autoradiography. Quantitation of ^{32}P-labeled proteins was determined after autoradiography by excision of the corresponding pp60^{F527}, enolase, or 120-kDa protein bands and counting in a liquid scintillation counter.

TABLE 1

***In vitro* inhibitory potency of tyrphostins**

The compounds were tested under the same conditions as described in the legend to Fig. 7. IC_{50} was defined as the drug concentration that reduced substrate phosphorylation by 50%.

Compound	IC_{50}		
	pp60^{F527} (autophosphorylation)	Enolase (exogenous substrate)	p120 (endogenous substrate)
	µM		
AG18	11	30	45
AG34	2	17	5
AG82	10	20	5

complete in treated cells. These results are in agreement with previous reports showing incomplete inhibition of EGF-R autophosphorylation or of its substrate phosphorylation in intact cells even at 100 µM AG18 or AG213 (18, 19). With respect to the observation that tyrphostins are "mixed" competitive inhibitors with respect to the substrate and ATP (10), one can imagine that the high intracellular concentration of ATP (2–3 mM) is in part responsible for this incomplete inhibition.

pp125^{FAK} has been identified as being one of the tyrosine-phosphorylated 120-kDa proteins in pp60^{F527}-expressing cells. This protein is a structurally distinctive protein tyrosine kinase associated with focal adhesions plaques (6). Immunoprecipitation experiments showed that the phosphorylation of pp125^{FAK} was virtually not inhibited by the tyrphostins in the treated pp60^{F527}-expressing cells. These results indicated that the protein of 120 kDa whose phosphorylation was inhibited in the anti-PTyr Western blot was not pp125^{FAK}. Because in Fig. 2a tyrosine-phosphorylated protein(s) still remained after the tyrphostin treatment, we proposed that one of these proteins could be pp125^{FAK}. It is unlikely that the protein whose phosphorylation was inhibited could be the p120 protein related to cadherin-binding factors β-catenin, plakoglobulin, and *Drosophila armadillo*, because mAb 2B12, raised against this protein, did not recognize any protein in our NIH/3T3 cells (24). p21^{ras}-GAP was not phosphorylated in our system, a result that is consistent with previous reports from Brott *et al.* (25) on v-*src*-transformed CEF cells. Ellis *et al.* (4) observed a high level of GAP phosphorylation in v-*src*-transformed Rat-2 cells. The discrepancies in GAP tyrosine phosphorylation could be linked to the different cellular systems used. The second substrate, p85, corresponds to cortactin, a protein characterized as being an actin-binding protein localized in the cell cortex (5, 8). All of the phosphorylated tyrosine residues in p85 were also retrieved in cortactin, suggesting that the subunit of phosphatidylinositol-3-kinase, which also has a size of 85 kDa and was phosphorylated by pp60$^{c\text{-}src}$ in the pp60$^{c\text{-}src}$/middle T antigen complex (26), was not or was very slightly phosphorylated in our system. The third substrate of pp60^{F527}, the p62 protein, was identified as being the p62 that coimmunoprecipitated with GAP in v-*src*-transformed cells (4, 7).

The effect on intact cells requires relatively long incubation times and high concentrations of tyrphostins. Previous reports have shown that incubation times of several hours are needed to observe an inhibitory effect on EGF-R tyrosine kinase activity with tyrphostins (19, 20). A possibility might be that turnover of prephosphorylated substrates must occur before the effect of the tyrphostins on *de novo* phosphorylation can be seen. This turnover would require several hours and would involve either proteolytic degradation of the substrates and/or dephosphorylation of the prephosphorylated substrates through tyrosine phosphatase activities.

We also showed that tyrphostins could inhibit pp60^{F527} kinase activity *in vitro*. The IC_{50} values ranged from 2 µM (AG34) to 45 µM (AG18). These values are very similar to those obtained for the EGF-R tyrosine kinase activity *in vitro*, suggesting that the affinities of the two enzymes for these inhibitors are very close (21, 23).

It has been shown that tyrphostins AG18 and AG213 did not alter the steady state level of the EGF-R (another tyrosine kinase) or its internalization after EGF binding, while inhibiting its activity (19). AG18 treatment did not affect any of the protein levels in our system, in agreement with what was reported previously (19). However, in AG34- and AG82-treated

pp60^{F527}-expressing cells, a 30 and 48% reduction, respectively, of this protein level was observed; under the same conditions, the levels of the src protein in control cells and the levels of the substrate proteins were unchanged, indicating a selective effect on the pp60^{F527} protein. The effects of AG34 and AG82 were at the translational and not the transcriptional level. None of the tyrphostins accelerated the rate of degradation of the protein. However, they all diminished the synthesis rate, with AG34 and AG82 being the most effective. Thus, at equilibrium, the steady state level of the pp60^{F527} protein was reduced only in the presence of AG34 and AG82. This effect of the tyrphostins within cells is a new mode of action. It reinforced their direct inhibitory action, because AG34 and AG82 were the most potent compounds in intact cells. However, this inhibitory effect on protein synthesis was not observed in an *in vitro* translation system. These results suggested that, in these cells, AG34 and AG82 might interact with one or more targets, resulting in the observed decrease of pp60^{F527} protein synthesis. Actually, the decrease of the steady state levels of the pp60^{src} proteins has already been observed with tyrosine kinase inhibitors. Several authors reported this effect for herbimycin A, another tyrosine kinase inhibitor, on pp60$^{v\text{-}src}$ in NRK cells or on pp60$^{c\text{-}src}$ in HT-29 cells (27, 28). With this inhibitor, the decrease of the src protein level has been related to specifically accelerated degradation.

The transformed phenotype of pp60^{F527}-expressing cells was reversed by treatment with the tyrphostins. AG82 and AG34, the most potent inhibitors of the protein kinase activity in intact cells, reversed this phenotype at lower concentrations (10 μM and 50 μM, respectively) after 72-hr exposure, compared with AG18 (100 μM). This strongly suggests that the phenotype reversion is directly linked to the reduction of the PTyr content induced by the tyrphostins in treated cells, in agreement with previous reports (28). It has been reported that cell transformation morphology is related to cytoskeletal protein modifications (29). Hence, we suggest that inhibition of the phosphorylation of one of the pp60^{F527} substrates, the cytoskeleton-associated p85 (cortactin), was important for this phenotype reversion. This is supported by data showing that AG18 and AG82 reversed the v-*src*-transformed phenotype of chick lens cells in parallel with inhibition of the tyrosine phosphorylation of cytoskeletal proteins (30).

In summary, our findings demonstrate an inhibitory effect of tyrphostins on pp60^{F527} tyrosine kinase activity. Some tyrphostins also decreased the steady state levels of the protein, an effect that potentiated their inhibitory actions. All of these tyrphostins could reverse the transformed morphology of the pp60^{F527}-expressing cells through the inhibition of the kinase activity.

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