Tyrphostin A23 Inhibits Internalization of the Transferrin Receptor by Perturbing the Interaction between Tyrosine Motifs and the Medium Chain Subunit of the AP-2 Adaptor Complex*

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Seventy intracellular membrane trafficking events are mediated by tyrosine-containing motifs within the cytosolic domains of integral membrane proteins. Many such motifs conform to the consensus YXXΦ, where Φ represents a bulky hydrophobic residue. This motif interacts with the medium chain (μ) subunits of adaptor complexes that link the cytosolic domains of integral membrane proteins to the clathrin coat involved in vesicle formation. The YXXΦ motif is similar to motifs in which the tyrosine residue is phosphorylated by tyrosine kinases. Tyrphostins (structural analogs of tyrosine) are inhibitors of tyrosine kinases and function by binding to the active sites of the enzymes. We previously showed that, in vitro and in yeast two-hybrid interaction assays, some tyrphostins can inhibit the interaction between YXXΦ motifs and the μ2 subunit of the AP-2 adaptor complex (Crump, C., Williams, J. L., Stephens, D. J., and Banting, G. (1998) J. Biol. Chem. 273, 28073–28077). A23 is such a tyrphostin. We now show that molecular modeling of tyrphostin A23 into the tyrosine-binding pocket in μ2 provides a structural explanation for A23 being able to inhibit the interaction between YXXΦ motifs and μ2. Furthermore, we show that A23 inhibited the internalization of 125I-transferrin in Hep7a cells without having any discernible effect on the morphology of compartments of the endocytic pathway. Control tyrphostins, active as inhibitors of tyrosine kinase activity, but incapable of inhibiting the YXXΦ motif/μ2 interaction, did not inhibit endocytosis. These data are consistent with A23 inhibition of the YXXΦ motif/μ2 interaction in intact cells and with the possibility that different tyrphostins may be used to inhibit specific membrane trafficking events in eukaryotic cells.

The trafficking and intracellular targeting of many transmembrane proteins depend on motifs found within their cytosolic domains. One such motif contains a critical tyrosine residue within the sequence YXXΦ, where Φ represents a bulky hydrophobic residue (1, 2). Tyrosine-based motifs conforming to this consensus sequence can interact directly with the medium chain (μ) subunits of heterotetrameric adaptor complexes involved in several different intracellular trafficking pathways. The AP-2 adaptor complex facilitates incorporation of transmembrane proteins containing YXXΦ motifs into clathrin-coated vesicles at the cell surface (1, 3–9), whereas AP-1, AP-3, and AP-4 adaptor complexes are involved in other intracellular vesicular transport steps (reviewed in Refs. 10–13). The μ subunits from all four adaptor complexes have been shown to interact with the YXXΦ motif, with the precise sequence and context of this motif determining the specificity of the interaction (14, 15). Mutation of residues within and around the YXXΦ motif affects interaction with μ subunits; but, in all cases, mutation of the tyrosine residue in the motif (e.g. to alanine) blocks interaction with the μ subunit (4, 6, 8). Structural studies on peptides containing these motifs have generated data that have been interpreted as showing that the YXXΦ motif exists as part of a tight turn, an α-helix, or a β-strand (16–20). However, whatever the predicted overall structure, each of these studies has shown the side chain of the critical tyrosine residue to project away from the peptide backbone.

The YXXΦ internalization motif is remarkably similar to sequences in which the tyrosine residue can be phosphorylated and, once phosphorylated, bind to Src homology 2 domains (21). It is now clear that although both tyrosine kinases and medium subunits of adaptor complexes recognize essentially the same motif, there is discrimination, such that very few tyrosine-based motifs that have been shown to interact with μ chains also act as substrates for tyrosine kinases (22–24). Furthermore, although both μ chains and tyrosine kinases accommodate the tyrosine side chain as part of their interaction with the YXXΦ motif, there is no great similarity between the YXXΦ-binding sites on μ chains and those on tyrosine kinases (20).

The similarity between motifs that interact with μ chains and those that are recognized by some tyrosine kinases suggested that structural analogs of the tyrosine side chain might disrupt the interaction between μ chains and their target tyrosine motifs. Tyrphostins are structural analogs of tyrosine. They were originally developed as substrate-competitive inhibitors of the epidermal growth factor tyrosine kinase (25–27). Tyrphostins have subsequently been used to investigate the physiological roles of many different tyrosine kinases. Some tyrphostins have also been reported to inhibit endocytosis and autophagy (28) and vesicle formation from the trans-Golgi network (29), thus implying a possible role for tyrosine kinases in these processes.

Our previous studies using tyrphostins involved the type I integral membrane protein TGN38, which cycles between the trans-Golgi network and the plasma membrane, but has a steady-state localization at the trans-Golgi network (reviewed in Ref. 30). The cytosolic domain of TGN38 contains a consensus tyrosine-based motif, SDYQRL, which is essential for internalization of the protein (31–34). Both this sequence and the complete cytosolic domain of TGN38 can specifically interact with the μ2 subunit of the plasma membrane-associated adapt-
tor complex AP-2 (4, 5, 8). Furthermore, this interaction is critically dependent on the tyrosine residue (4, 8). We previously used an in vitro assay and yeast two-hybrid growth assays to show that tyrphostins A23 and A46 inhibit the interaction between TGN38 and μ2 (35). However, a range of other tyrphostins did not disrupt the interaction, demonstrating that the effect was independent of tyrosine kinase inhibition.

We have now modeled the structure of A23 and other tyrphostins into the YXXΦ-binding cleft in μ2. This has provided us with a structural explanation for the inhibition of the YXXΦ motif/μ2 interaction by A23.

If tyrphostin A23 were to block the interaction between YXXΦ motifs and μ2 in intact cells, we would predict that clathrin-mediated endocytosis would be inhibited. We have now tested this by following the internalization of transferrin (TF) in cells incubated in the presence or absence of various tyrphostins. TF is internalized upon binding to its receptor at the cell surface. This receptor is internalized via a clathrin-mediated pathway by virtue of an interaction between a tyrosine-based motif, YTRF, and μ2 (16, 36). Thus, inhibition of clathrin-mediated endocytosis leads to a reduction in internalized TF (for example, see Ref. 37). We now show that tyrphostin A23 inhibits the internalization of the TF receptor (TFR) from the plasma membrane. This effect is specific for A23; other tyrphostins that act as inhibitors of tyrosine kinase activity do not inhibit TF internalization. Thus, this inhibition of TF internalization in intact cells correlates with the capacity of A23 to inhibit the interaction between YXXΦ motifs and μ2 in vitro.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling**—The crystal structure (Protein Data Bank Code 1i31) of the μ2 adaptin subunit (AP50) of the AP-2 clathrin adaptor complexed with the peptide FYRALM (20) was used as the starting point for modeling. Hydrogen atoms were added to the structure consistent with pH 7. All residues with an atom within 25 Å of lysine 203 were selected from the structure, and the rest were discarded. The remaining volume in this sphere was filled with water, and all residues in the concentric sphere between 20 and 25 Å were fixed in space during subsequent calculations. Those residues in the 20-Å sphere were energy-minimized to an average derivative of 0.01 kcal/Å. Partial charges for the Tyrphostins (Sigma and Calbiochem) were stored as 3,5,4-dihydroxyphenyl compounds (as described by Harlow and Lane (39)) and examined on an inactivated Leica TCS NT (UV) confocal laser-scanning microscope with a ×100 plan apo objective lens. Care was taken to ensure equal laser intensity, photomultiplier tube setting, and pinhole size for TF internalization assays. Optical slices were taken at 0.5- or 1-μm intervals through the cells and displayed as maximum projections in the microscope software. The images were then imported to Adobe Photosho Version 6.0 to produce the final figures.

Mean pixel intensity measurements were determined over four quadrants of cells at zoom ×1 and magnification ×63 using the Leica confocal microscope software. The data were processed with Microsoft Excel and plotted with SigmaPlot 2001 (Version 7.1) using the linear regression function.

**Transferrin Receptor Degradation Assay**—Heb7a cells were incubated in the presence of 350 μM tyrphostin A23 for 0, 15, or 30 min. Post-nuclear cell lysates were prepared (40), and equal amounts of protein, as determined by the Bradford assay (41), were electrophoresed under reducing conditions on a 10% SDS-polyacylamide gel. The gel was immunoblotted with a murine monoclonal antibody (H68.4) raised against the cytosolic domain of the human TIR (a gift from C. R. Hopkins) (42).

**RESULTS**

**Molecular Modeling of Tyrphostins into the μ2 Tyrosine-binding Pocket**—We have previously shown that, in vitro, both tyrphostins A8 and A23 can inhibit the interaction between YXXΦ motifs and μ2 in recombinant protein interaction assays, whereas tyrphostins A47, A25, and A51 are not (35). We observed that the phenyl rings of tyrphostins A8, A23, A46, and A47 were accommodated within the tyrosine-binding cleft in μ2, whereas those of tyrphostins A25 and A51 were not. Tyrphostins A8 and A63 are monohydroxylated at position 4 of the phenyl ring in their structure (i.e. the same as a tyrosine side chain); tyrphostins A23, A25, and A47 are 3,4-dihydroxylated on their phenyl ring; and tyrphostins A25 and A51 are 3,4,5-trihydroxyphenyl compounds (Fig. 1). The energy-minimized complexes of A8 and A23 in orientation/conformation P (see “Experimental Procedures”) resulted in the tyrphostins binding snugly in the pocket occupied by tyrosine in the FYRALM peptide complex. The O⁻ atom forms a hydrogen-bonding network with Lys203, Asp176, and Arg423 at the base of the binding cleft, whereas the phenyl ring lies in the hydrophobic groove formed by the side chain of Arg423, Phe174, and Trp421, as shown in Fig. 2. In contrast, the complexes in orientation/conformation Q (see “Experimental Procedures”) break this hydrogen-bonding network because steric clashes between the dinitrile group and Val422 force the tyrphostins — 2 Å along the groove, away from the O⁻-binding site. In the P complex of tyrphostin A23, the second ring oxygen abuts the guanidinium group of Arg423. This probably accounts for the fact that this compound is more efficient than tyrphos-
tamin A8 as an inhibitor of the interaction between YXXΦ motifs and μ2 (35). The reason for the failure of tyrphostins with three-ring hydroxy groups (e.g., A25 and A51) to inhibit the interaction between YXXΦ motifs and μ2 also becomes evident from inspection of Fig. 2. The third hydroxy group would necessarily be forced into the hydrophobic part of the cleft (lined by Leu175) if the compound were to bind in a similar fashion to that proposed for A8 and A23. Thus, it appears that the tyrosine-binding cleft in μ2 can accommodate a 3,4-dihydroxy derivative, but not a 3,4,5-trihydroxy derivative, of a phenyl ring. This is consistent with our previous in vitro observations on the inhibition of YXXΦ motifs by tyrphostins (35). In fact, addition of an extra hydroxyl group in the 3-position of the phenyl ring is beneficial for the interaction with μ2 (see above). Thus, a 3,4-dihydroxyphenyl compound (such as tyrphostin A23 or A46) is predicted, by molecular modeling studies, to both fit well in the tyrosine-binding cleft of μ2 and be stabilized in that binding by hydrogen bonding and other interactions (Fig. 2). This provides an explanation for our earlier observation (35) that tyrphostin A23 inhibits the interaction between μ2 and a YXXΦ motif more efficiently than tyrphostin A8 (which is structurally identical to tyrphostin A23) apart from the fact that A8 is a 4-monohydroxyphenyl compound and A23 is a 3,4-dihydroxyphenyl compound (Table I). However, these data do not explain why tyrphostin A63 should not be equally as efficient as tyrphostin A8 in inhibiting the interaction between μ2 and a YXXΦ motif because both are 4-monohydroxyphenyl compounds (Fig. 1). In fact, the only difference between the structures of tyrphostins A8 and A63 is that the former has a double bond linking the malononitrile group to the aromatic ring, whereas the latter does not (Fig. 1).

This led us to suspect that the pK_a values for the hydroxyl groups on the phenyl rings of the two molecules might be different. Measurements of the tyrphostin pK_a values showed this to be the case, with the pK_a for tyrphostin A8 being 6.73 and that for A63 being 9.61 (Table I). This implies that the tyrphostin anion is the species that binds into the μ2 active site and complements the overall positive charge at the base of the cleft, which is composed of the side chains of Lys203, Asp176, and Arg423 (Fig. 2).

Tyrphostins A46 and A47 are structurally very similar, both being 3,4-dihydroxyphenyl compounds, and both can be modeled into the tyrosine-binding cleft in μ2. Furthermore, the pK_a values for A46 and A47 (7.13 and 6.87, respectively) (Table I) are such that these compounds will be mostly ionized at physiological pH. However, our previous in vitro binding studies showed that only tyrphostin A46 efficiently inhibits the interaction between μ2 and a YXXΦ motif (35). Because the structure and acidity of these tyrphostins are so similar, it was unclear why A47 was inactive, whereas A46 showed good activity. A possible explanation lies in the relative stability of these tyrphostins toward hydrolysis. Tyrphostins A46 and A47 were incubated in the dark in pH 7 buffer at 37 °C, and the absorbance due to the anion was measured at regular intervals.

**Table I**

| Tyrophostin | λ | pK_a |
|------------|---|------|
| A8         | 416 | 6.73 ± 0.03 |
| A23        | 435 | 6.53 ± 0.06 |
| A46        | 420 | 7.13 ± 0.03 |
| A47        | 470 | 6.87 ± 0.03 |
| A63        | 293 | 9.61 ± 0.02 |

![Fig. 1. Structures of tyrphostins considered in this study.](image1)

![Fig. 2. Molecular modeling of tyrphostins A8 and A23 into the tyrosine-binding cleft in μ2. Energy-minimized complexes of A8 and A23 (large ball and stick) with μ2 adaptin (small ball and stick). All protein residues within 5 Å of the ligands are shown. Oxygen and nitrogen atoms are shown as dark gray spheres, and carbon atoms as light gray spheres.](image2)
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125I-Transferrin Internalization Assays—Having established the structural basis for the inhibition of the YXXØ motif/µ2 interaction by tyrphostin A23, it was pertinent to investigate whether this inhibition occurs in vivo to inhibit trafficking pathways in a mammalian system. To address this question, we focused on the internalization of Tf from the plasma membrane. Tf is internalized following binding to the TfR at the cell surface, with the internalization of the TfR being dependent upon interactions between µ2 and the tyrosine-based internalization motif (YTRF) in the TfR cytosolic domain (16, 36, 44). A biochemical assay was used to probe the effects of selected tyrphostins on Tf internalization. The assay followed the internalization of 125I-Tf into Heb7a cells incubated at 37 °C in the presence or absence of different tyrphostins (Fig. 4). Incubation of cells in the presence of 350 µM tyrphostin A23 (the concentration selected as a result of observed effects in in vitro assays (35)) for 30 min led to a marked decrease in the rate (−4-fold) of 125I-Tf internalization compared with that observed in control cells (Fig. 4A). Control tyrphostins (A51 and A63, previously shown to have no effect on the interaction between µ2 and the TGN38 YXXΦ internalization motif (35)) had no significant effect on 125I-Tf internalization (Fig. 4, B and C).

Tyrphostins A51 and A23 (IC50 for inhibition of epidermal growth factor receptor tyrosine kinase activity of 0.8 and 35 µM, respectively) were both efficient inhibitors of tyrosine kinase activity, but only A23 inhibited Tf internalization (Fig. 4, compare A and B). Tyrphostin A63 (IC50 for inhibition of epidermal growth factor receptor tyrosine kinase activity of 6500 µM) has been used as a negative control in assays of inhibition of tyrosine kinase activity by tyrphostins (27); it was also ineffective as an inhibitor of endocytosis (Fig. 4C).

Tyrphostins A23 and A51 were both used at 10 times their IC50 values for inhibition of epidermal growth factor receptor tyrosine kinase activity in these assays. The fact that only A23 inhibited Tf internalization indicates that it exerts its effect by a mechanism other than inhibition of tyrosine kinase activity. Given our previously published in vitro data (35), this mechanism is most
likely the inhibition of the interaction between μ2 and the YTRF internalization motif in the cytosolic domain of the TfR.

Internalization of Fluorescently Labeled Transferrin—Human serum Tf conjugated to Alexa 594 (Alexa 594-Tf) was also used as a tool to investigate any possible effects of tyrphostin A23 on trafficking pathways (Fig. 5). Heb7a cells were treated with 350 μM tyrphostin A23 for varying times and allowed to internalize Alexa 594-Tf. Cells exposed to tyrphostin A23 for 30 min in total (B) showed reduced internalization of the fluorescent probe compared with untreated cells (A). Heb7a cells exposed to tyrphostin A23 for longer periods (C–F) showed no significant morphological changes in the distribution of the fluorescent probe compared with control cells (A). Scale bar = 10 μm.

These results suggest that although the rate of Tf internalization was inhibited by tyrphostin A23, this tyrphostin had no significant effect on the distribution of Alexa 594-Tf throughout the recycling pathway over the time course of the experiment. Alexa 594-Tf uptake experiments were also performed on Heb7A cells incubated in the presence of different concentrations of tyrphostin A23 for 30 min. The inhibitory effect of tyrphostin A23 on Alexa 594-Tf uptake was found to be dose-dependent (Fig. 6).

Internalization of TGN38—Because our original in vitro studies focused on the effects of tyrphostins on the interaction between μ2 and the tyrosine-based internalization motif in the cytosolic domain of TGN38 (35), the effect of A23 on the internalization of TGN38 was also assayed. This was done by an
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Fig. 7. Immunoﬂuorescence of endomembrane markers in Heb7a cells treated with tyrphostin A23. Heb7a cells were incubated in the presence (A, C, E, and G) or absence (B, D, F, and H) of 350 μM tyrphostin A23 for 30 min. The cells were ﬁxed with methanol (A–F) or paraformaldehyde (G and H) and labeled with antibodies raised against LAMP-1 (CD107a) (A and B), LAMP-2 (CD107b) (C and D), CD63 (E and F), and EEA1 (G and H). Alexa 488-conjugated secondary antibodies were used for detection. Cells were imaged as described in the legend to Fig. 5. Cells exposed to tyrphostin A23 for 30 min (A, C, E, and G) showed no signiﬁcant morphological changes in the distribution of the endomembrane markers compared with control cells (B, D, F, and H). Scale bar = 10 μm.

antibody uptake assay using a monoclonal antibody that recognizes the extreme N terminus of TGN38 (2F7.1), followed by detection of the internalized antibody using a ﬂuorescently labeled secondary antibody as described previously (34). Normal rat kidney cells were incubated in the presence or absence of 350 μM A23 for 5 min at 37 °C and then with antibody 2F7.1 in the continued presence or absence of 350 μM A23 for 30 min at 37 °C prior to methanol ﬁxation, incubation with secondary antibody, and processing for ﬂuorescence microscopy. Mean pixel intensities were recorded from both sets of cells under identical imaging conditions (as described under “Experimental Procedures”). The mean pixel intensity (in arbitrary units) in control cells was 121.25 ± 22.10 (n = 50 cells), and that in A23-treated cells was 38.09 ± 10.05 (n = 50 cells). Thus, in addition to inhibiting Tf uptake, A23 also inhibited the internalization of TGN38. No other tyrphostin tested had any effect on the internalization of TGN38.

Internalization of Fluorescent Dextran—Our earlier in vitro assays (35) and the modeling studies presented here suggest that the inhibition of Tf uptake and of TGN38 internalization by A23 is a result of inhibition of the interaction between μ2 and the tyrosine-based internalization motifs in the cytosolic domains of the TIR and TGN38. If this is the case, then internalization of a ﬂuid-phase marker should not be affected by incubation of cells in the presence of A23. To address this, Heb7a cells were incubated in the presence of 1 mg/ml ﬂuorescently labeled 10-kDa dextran (as described under “Experimental Procedures”) for 30 min at 37 °C in the presence or absence of 350 μM A23. Mean pixel intensities were then recorded from both sets of cells under identical imaging conditions. The mean pixel intensity (in arbitrary units) in control cells was 34.0 ± 9.9 (n = 30 cells), and that in A23-treated cells was 38.0 ± 11.0 (n = 30 cells). Thus, incubation of cells in the presence of A23 for 30 min at 37 °C had no effect on ﬂuid-phase uptake of dextran. No other tyrphostin tested had any effect on ﬂuid-phase uptake of dextran.

Analysis of the Morphology of Endocytic Compartments—The fact that the distribution of internalized Alexa 594-Tf in cells that had been incubated in the presence of tyrphostin A23 was indistinguishable from that in control cells suggested that the compartments of the endocytic recycling pathway were not perturbed by the presence of tyrphostin A23. To conﬁrm this and to address possible effects of tyrphostin A23 on the morphology of other endocytic compartments, we used immunoﬂuorescence microscopy to localize a range of endomembrane markers in both A23-treated (350 μM, 30 min) and untreated Heb7a cells. No signiﬁcant changes in the localization of the lysosomal/late endosomal markers LAMP-1 (Fig. 7, A and B), LAMP-2 (C and D), and CD63 (E and F) and the early endosomal marker EEA1 (G and H) were observed. Similar analysis using markers for the Golgi apparatus and trans-Golgi network also failed to show any difference between control cells and those that had been incubated with tyrphostin A23 (data not shown).

Transferrin Receptor Degradation Assay—We considered it possible that, although there were no gross changes in the morphology of endocytic compartments, the presence of tyrphostin A23 might affect the internalization/recycling of TfRs...
such that an elevated proportion of internalized TRs might be routed to late endosomes and lysosomes for degradation. This possibility was investigated by immunoblot analysis of post-nuclear supernatants from Hep7a cells that had been incubated in the presence of 350 μM tyrphostin A23 for different times (Fig. 8). The blot was probed with a murine monoclonal antibody (H68.4) (42) raised against the cytosolic domain of the human TR. No reduction in the level of TR expression was observed over the time course of the experiment. These results are consistent with tyrphostin A23 inhibition of the interaction between the tyrosine-based internalization motif on the TR and the μ2 medium chain subunit of the AP-2 adaptor complex. These data show that tyrphostin A23 can be used as an inhibitor of endocytosis in mammalian cells and that, when using tyrphostins as inhibitors of tyrosine kinases, any observed effects on membrane trafficking events should be interpreted with caution due to possible interaction with μ chains. It is possible that different structural analogs of tyrphostin could inhibit the interaction between tyrosine-based motifs and other adaptor complex medium chains. Future molecular modeling could potentially identify new drugs designed to inhibit other membrane trafficking pathways. These drugs could target different adaptor complex medium chains or their as yet unidentified homologs. Thus, specifically designed tyrphostins may become useful tools for dissecting membrane trafficking pathways in eukaryotic cells.

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