Inhibition of the Interaction between Tyrosine-based Motifs and the Medium Chain Subunit of the AP-2 Adaptor Complex by Specific Tyrphostins*

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Several intracellular membrane trafficking events are mediated by tyrosine-containing motifs found within the cytosolic domains of certain integral membrane proteins. Many of these tyrosine motifs conform to the consensus YXXΦ (where Φ represents a bulky hydrophobic residue). This YXXΦ motif has been shown to interact with the medium chain subunits of adaptor complexes that generally link relevant integral membrane protein cytosolic domains to the clathrin coat involved in vesicle formation. The motif YXXΦ is also very similar to motifs that are targets for phosphorylation by tyrosine kinases. Tyrosine kinase inhibitors known as tyrphostins are structural analogues of tyrosine, and so it is possible that tyrphostins could also inhibit interactions between medium chains and YXXΦ motifs. TGN38 is a type I integral membrane protein containing a tyrosine motif, YQRL, within the cytosolic domain. We have previously shown that this motif interacts directly with the medium chain subunit of the plasma membrane localized AP-2 adaptor complex (μ2). We have investigated a range of tyrphostins and demonstrated a specific inhibition of the interaction between μ2 and the TGN38 cytosolic domain by tyrphostin A23 through in vitro analysis and the yeast two-hybrid system. These data raise the exciting possibility that different membrane traffic events could be inhibited by specific tyrphostins.

The trafficking and intracellular targeting of many transmembrane proteins relies upon various signal motifs found within the cytosolic domains of these proteins. One of the best characterized of these targeting motifs centers around a critical tyrosine residue often found within the sequence YXXΦ (where Φ represents a bulky hydrophobic residue) (1–3). Such interactions have been shown for both the medium chain subunits of AP-1 and AP-2, the trans-Golgi network (TGN), and at least some tyrosine kinases. We have also previously shown that this sequence and the complete cytosolic domain of TGN38 can specifically interact with μ2, the medium chain subunit of the plasma membrane-associated clathrin adaptor complex, and that this interaction is critically dependent on the tyrosine residue (3, 7). Due to the similarity between these recognition motifs, we were interested in determining whether any tyrphostin tyrosine kinase inhibitors could inhibit the interaction of TGN38 and μ2. The cytosolic domain of TGN38 is a particularly suitable sequence for such a study because we have already shown that the tyrosine residue in the SDYQRL motif in the cytosolic domain of TGN38 can be phosphorylated in vitro by the insulin receptor tyrosine kinase, whereupon it can bind to the SH2 domains of Syk (15). We have utilized an in vitro assay to test a range of tyrphostins and have shown a strong inhibition of the interaction between TGN38 and μ2.

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and μ2 by tyrphostins A23 and A46. We have also demonstrated a strong inhibition of the interaction between TGN38 and μ2 by tyrphostin A23 in yeast two-hybrid growth assays.

**EXPERIMENTAL PROCEDURES**

All reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

**Far Western Blot Interaction Assay**—Soluble fusion proteins of the C-terminal two-thirds of μ2 (fused to thioredoxin; TRX-μ2ΔN) and the wild type or Y333A mutant cytosolic domain of TGN38 (fused to glutathione S-transferase; GST-TGN38wt and GST-Y333A) were expressed and purified as described previously (7). The C-terminal two-thirds of μ2 was used because this portion of μ2 has previously been shown to be the domain responsible for interaction with tyrosine-based motifs, and the solubility of the protein is improved by deletion of the N terminus (6, 28). 5 μg of GST-TGN38wt, GST-Y333A, or GST alone were immobilized on nitrocellulose membrane strips by slot blotting, and the membranes were incubated in three changes of blocking buffer (Tris-buffered saline, 0.02% v/v Tween 20, 10% v/v dried milk powder) for 1 h at room temperature. Membranes were incubated with 10 μg of TRX-μ2ΔN in the presence or absence of different tyrphostins (at concentrations of 1 order of magnitude above and 1 order of magnitude below the commercially reported IC_{50} concentration for tyrosine kinase inhibition) in 1 ml of blocking buffer for 90 min at room temperature. Membranes were washed in six changes of washing buffer (Tris-buffered saline, 0.02% v/v Tween 20) for 1 h at room temperature, incubated with monoclonal anti-thioredoxin antibodies (Invitrogen), 1/2500 in blocking buffer for 30 min at room temperature, washed in six changes of washing buffer for 1 h at room temperature, and developed using the chemiluminescence detection system (Boehringer Mannheim).

**Yeast Two-hybrid Growth Assay**—Yeast cells (strain L40 (29)) were co-transformed with vectors encoding the wild type TGN38 cytosolic domain fused to the LexA DNA binding domain (pBTM116-TGN38wt) and μ2 fused to the VP16 transactivating domain (pVP16-μ2) or pVP16 alone, or co-transformed with a positive control vector encoding the LexA DNA binding domain fused to the VP16 transactivating domain (pLexA-VP16) as described previously (7). Quantitative growth assays were performed as described previously (28). Briefly, overnight cultures of each yeast strain were grown at 30 °C in synthetic yeast medium containing histidine to allow all strains to grow to saturation. 10-ml aliquots of fresh synthetic medium lacking histidine were inoculated with 5 × 10^{-7} A_{600} units of each saturated overnight culture in triplicate, in the presence or absence of tyrphostins (at a concentration of 1 order of magnitude above the reported IC_{50} for tyrosine kinase inhibition). Cultures were incubated at 30 °C for 72 h, and A_{600} measurements were taken at the intervals shown in the figure legends. 3-Ami-no-1,2,4-triazole (3-AI) inhibition curves were generated by inoculating 2.5 × 10^{-7} A_{600} units of the appropriate saturated overnight yeast cultures into triplicate 5 ml of synthetic medium lacking histidine, supplemented with 0, 0.1, 1, 10, or 100 mM 3-AT, and incubated at 30 °C in the presence or absence of the appropriate tyrphostins (at a concentration 1 order of magnitude above the reported IC_{50} for tyrosine kinase inhibition), and A_{600} measurements were taken after 48 h. Repeat additions of appropriate tyrphostins (the same amount as originally added to each culture) were added to the yeast cultures every 24 h to counteract degradation of the tyrphostins.

**RESULTS**

Previous work has shown that the medium chain (μ) sub-units of clathrin-associated adaptor complexes can interact with tyrosine residues found within the motif YXXΦ (where Φ = any amino acid and Φ = bulgy hydrophobic residue) (3–9). Tyrosine residues that can be phosphorylated and bind to SH2 domains can also be found in remarkably similar sequence motifs, i.e. YXXΦ (14). It has been reported that a single tyrosine motif can act as both a μ chain binding region and as a target for a tyrosine kinase and subsequent SH2 domain binding (16, 17), but this is the only evidence for such an occurrence in *vivo* to date. However, peptides corresponding to this motif where the tyrosine is in the phosphorylated form no longer show binding to μ chains (4, 6). Due to the similarity of the motifs that interact with μ chains and some tyrosine kinases, we were interested in whether a family of structural analogues of the tyrosine side chain (tyrphostins, tyrosine kinase inhibitors) can disrupt the interaction between μ chains and their target tyrosine motifs.

**Far Western Blot Assays**—We utilized a far Western blot procedure to analyze the effect of a range of tyrphostin chemicals on the interaction of the cytosolic domain of TGN38 containing the motif SDYQRQL and the AP-2 adaptor complex medium chain (μ2). Of all tyrphostins tested, only A23 and A46 showed a complete inhibition of the TGN38-μ2 interaction at a concentration 10 times above the reported IC_{50} for inhibition of the epidermal growth factor receptor kinase (Fig. 1). Virtually all other tyrphostins at the same relative concentrations (with respect to the reported IC_{50} for tyrosine kinase inhibition) demonstrated no inhibition of the TGN38-μ2 interaction, with signals the same as control levels (data for tyrphostins A1 and A47 shown in Fig. 1). Tyrophostins A8 and A25 demonstrated a slight inhibition of TGN38-μ2 interaction at concentrations 10 times above their IC_{50}, with the interaction signal being weaker than control levels but not abolished (data not shown).

![Fig. 1. Inhibition of the interaction between TGN38 cytosolic domain and μ2 in a far Western blot assay.](image-url)

**Yeast Two-hybrid Growth Assays**—The yeast two-hybrid system was utilized to further test the inhibition of the TGN38-μ2 interaction. Yeast expressing two proteins that interact, one fused to a DNA binding domain and one fused to a transactivating domain, can drive the expression of various reporter genes. One such reporter gene is the HIS3 gene that allows the
growth of the yeast strain in the absence of histidine. The affinity of the interaction between two proteins is proportional to the rate of growth of the expressing yeast in the absence of histidine, and thus growth curves can be used to analyze the strength of an interaction between two proteins. Yeast expressing the TGN38 cytosolic domain fused to the LexA DNA binding domain and μ2 fused to the VP16 transactivating domain were grown in the presence or absence of various tyrphostins, at a concentration 10 times above the reported IC50 of tyrosine kinase inhibition. The rate of growth was followed by measuring the optical density at 600 nm of the cultures at various time points. Yeast cells expressing the positive control construct, LexA fused to VP16, thus acting as a permanent interaction, were also grown under the same conditions, and the growth rate was determined. TGN38-μ2 yeast cultures demonstrated a significantly slower rate of growth in the presence of tyrphostin A23 (Fig. 2A), whereas positive control LexA-VP16 yeast cells were largely unaffected by the same treatment (Fig. 2B). This shows that tyrphostin A23 can inhibit the interaction of TGN38 with μ2 in living yeast cells. No other tyrphostin tested demonstrated inhibition of the TGN38-μ2 interaction (data not shown). This included A46, which showed a strong inhibition in the far-Western blot assay. This was surprising considering the inhibition demonstrated by A23 in the growth assays but may be due to yeast cells being able to either exclude growth inhibition assays—The affinity of an interaction between two proteins in the yeast two-hybrid system can also be demonstrated by using growth inhibition assays. 3-AT is an inhibitor of the yeast His3 gene product and therefore inhibits growth of histidine prototrophs that arise due to the TGN38-μ2 interaction. A higher affinity interaction between the TGN38 cytosolic domain and μ2 in the two-hybrid system leads to a higher expression level of the His3 gene, and so a greater concentration of 3-AT is required to inhibit the growth of these yeast cultures. Yeast expressing the LexA-TGN38 and μ2-VP16 fusion proteins were grown in -His liquid medium in the presence or absence of tyrphostin A23 and growth levels determined by A600 readings at the time points shown. Growth rates depend on the expression level of the His3 gene product that is proportional to the affinity of interaction between separately expressed LexA and VP16 fusion proteins. A, LexA-TGN38 + μ2-VP16 expressing yeast, +A23 (○) and −A23 (●). B, LexA-VP16 expressing yeast, +A23 (○) and −A23 (●). All A600 readings are the means ± S.D. of triplicate results. Where not shown, error bars lie within the data points.

Table I

| Tyrphostin | Reported IC50 for tyrosine kinase inhibition (μM) | Inhibition of TGN38-μ2 interaction | Structure |
|------------|-----------------------------------------------|---------------------------------|-----------|
| A1         | >1259                                         | −                               |           |
| A8         | 45                                            | +                               |           |
| A23        | 40                                            | +++                             |           |
| A25        | 3                                             | +                               |           |
| A46        | 10                                            | +++                             |           |
| A47        | 2.4                                           | −                               |           |
| A51        | 0.8                                           | −                               |           |
| A63        | 6500                                          | −                               |           |
| AG126      | ~10                                           | −                               |           |
| AG1288     | ~10                                           | −                               |           |
| AG1295     | 0.5                                           | −                               |           |
| AG1296     | 1                                             | −                               |           |

FIG. 2. Tyrphostin A23 inhibits the growth of yeast expressing LexA-TGN38 and μ2-VP16 but not yeast expressing LexA-VP16. Yeast transformants were grown in -His liquid medium in the presence or absence of tyrphostin A23 and growth levels determined by A600 readings at the time points shown. Growth rates depend on the expression level of the His3 gene product that is proportional to the affinity of interaction between the separately expressed LexA and VP16 fusion proteins. A, LexA-TGN38 + μ2-VP16 expressing yeast, +A23 (○) and −A23 (●). B, LexA-VP16 expressing yeast, +A23 (○) and −A23 (●). All A600 readings are the means ± S.D. of triplicate results. Where not shown, error bars lie within the data points.

A46 from their cytosol or rapidly metabolize A46 to an inactive form.
Tyrosine kinases can phosphorylate tyrosine residues in the cytosolic domains of many transmembrane proteins (3–8). Various tyrosine kinases can phosphorylate tyrosine residues within the cytosolic domains of many transmembrane proteins (3–8). Furthermore, various tyrosine kinases can phosphorylate tyrosine residues found within the cytosolic domains of many transmembrane proteins (3–8). The observation that μ chains and at least some tyrosine kinases can recognize very similar motifs leads to the possibility that both μ chains and tyrosine kinases may have similar binding pockets for the target tyrosine. Structural studies on peptides containing the YXXΦ motif from proteins internalized from the cell surface via AP-2-associated clathrin-mediated endocytosis have shown that the side chain of the critical tyrosine residue is exposed either in a tight turn or as part of an α-helix (10–13).

Structural analogues of tyrosine, known as tyrphostins, have been widely used over the last 10 years as potent tyrosine kinase inhibitors in numerous studies (18–21). Because tyrphostins presumably inhibit tyrosine kinases by competitive interaction with the tyrosine binding pocket, it is possible that these same chemicals could inhibit the interaction of μ chains with their target YXXΦ sequences in the same manner. However, as yet there are no published reports of the effect of these tyrosine analogues on the interaction of YXXΦ motifs with adaptor complex μ chains.

We have demonstrated a potent specific inhibition of the in vitro interaction between the cytosolic domain of TGN38 (containing the motif SDYQRL) and the medium chain subunit of the AP-2 complex, μ2, by two tyrphostins, A23 and A46. All other tyrphostins tested showed little or no effect on the level of in vitro interaction between TGN38 and μ2. Comparison of the structures of the tyrphostin chemicals shows very specific structural features of the tyrphostins required for the inhibition of the TGN38-μ2 interaction (Table I). Both tyrphostins A23 and A46 have hydroxyl groups in positions 3 and 4 of the phenyl ring with A46 containing an amide group in place of one of the nitrile groups found in A23 (A23 = α-cyano-(3, 4-dihydroxy)-cinnamitrile, A46 = α-cyano-(3,4-dihydroxy)-cinnamide). However, tyrphostins A8 and A25 have almost identical structures to A23 but with 4-monohydroxylated or 3,4,5-trihydroxylated phenyl rings, respectively (A8 = α-cyano-(4-hydroxy)-cinnamitrile, A25 = α-cyano-(3,4,5-trihydroxy)-cinnamitrile) but show only slight or no inhibition of the TGN38-μ2 interaction. Also tyrphostin A47 has a very similar structure to A46 with the oxygen atom of the cinnamid substituent with a sulfur atom to give a thiocinnamide (A47 = α-cyano-(3,4-dihydroxy)-thiocinnamide), but A47 also shows no inhibition of the TGN38-μ2 interaction.

We have also shown a significant inhibition of TGN38-μ2 interaction by tyrphostin A23 in the yeast two-hybrid system. These results support the observed in vitro data, demonstrating the same reproducible inhibition in a different environment. The environment in which the protein-protein interaction occurs in the yeast two-hybrid system is more reminiscent of in vivo conditions, suggesting that the observed inhibition in the far Western blot analysis is not an artifact of a purely in vitro assay. The inhibition of the interaction in the yeast two-hybrid system is signified by an inhibition of the growth of the yeast cultures, and so it is conceivable that the growth inhibition was due to inhibition of some essential tyrosine kinase within the yeast cells. However, this seems unlikely due to the fact that the positive control yeast cultures showed a very similar growth curve irrespective of the presence of tyrphostin A23. Also other tyrphostins tested showed no inhibition of yeast growth even though they were at the same relative concentrations with respect to the reported IC50 values for tyrosine kinase inhibition. The fact that tyrphostin A46 showed no inhibition of the TGN38-μ2 interaction in the yeast two-hybrid assays was surprising considering the potent inhibition observed in the in vitro analysis. However, this could be due to several factors. The yeast cells could be able to exclude A46 but
not A23 from their cytosol or efficiently metabolize A46 but not A23. It should be noted that even A47, which differs from A46 only in the fact that the oxygen atom of the cinnamide group has been substituted by a sulfur atom, fails to inhibit the interaction between A23 and TGN38 cytosolic domain, but this same component had no effect on A23.

In summary our data show that at least one tyrphostin appears to be able to specifically inhibit the interaction of a tyrosine-based motif with the AP-2 medium chain subunit, μ2. As far as we are aware this has never been demonstrated before. Furthermore it is conceivable that these tyrphostins or other structural analogues of tyrosine could inhibit the interaction between tyrosine-based motifs and the other adaptor complex medium chains and possibly as yet unidentified medium chain homologues. This observation could lead to the possibility of inhibitor chemicals being designed based around the tyrphostin backbone that could inhibit specific membrane traffic pathways. Previous studies with tyrphostins have often shown potent inhibition of membrane traffic events at concentrations lower than those used in this study, and thus the previous data still indicate that tyrosine kinases play a role in the membrane traffic events studied. However, the results presented here suggest that caution should be used in future interpretation of the effects of tyrphostins on membrane traffic events due to the possible inhibition of μ chain interactions. Hopefully these data will lead to further research into the effect of these chemicals on membrane traffic in eukaryotic cells.

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