A new type of protein methylation activated by tyrphostin A25 and vanadate

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Abstract **It has been reported that *S*-adenosylmethionine-dependent protein methylation in rat kidney extracts can be greatly stimulated by tyrphostin A25, a tyrosine kinase inhibitor. We have investigated the nature of this stimulation. We find that addition of tyrphostin A25, in combination with the protein phosphatase inhibitor vanadate, leads to the stimulation of methylation of polypeptides of 64, 42, 40, 36, 31, and 15 kDa in cytosolic extracts of mouse kidney. The effect of tyrphostin appears to be relatively specific for the A25 species. The enhanced methylation does not represent the activity of the families of protein histidine, lysine or arginine methyltransferases, nor that of the L-isoaspartyl/D-aspartyl methyltransferase, enzymes responsible for the bulk of protein methylation in most cell types. Chemical and enzymatic analyses of the methylated polypeptides suggest that the methyl group is in an ester linkage to the protein. In heart extracts, we find a similar situation but here the stimulation of methylation is not dependent upon vanadate and an additional 18 kDa methylated species is found. In contrast, little or no stimulation of methylation is found in brain or testis extracts. This work provides evidence for a novel type of protein carboxyl methylation reaction that may play a role in signaling reactions in certain mammalian tissues.**
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Keywords: Carboxyl methylation; Protein methylation; Tyrphostin; Vanadate

1. Introduction

Enzymatic protein methylation reactions can be used by cells to expand the functional and structural diversity of the 20 amino acids used in ribosomal protein synthesis and to modulate their activity [1]. Of special interest in understanding regulatory processes are potentially reversible reactions in which *S*-adenosyl-L-methionine is used as the methyl donor to form protein methyl esters. Four distinct classes of these carboxyl methyltransferases, distinguished by the amino acid residue that they modify, have been described to date, and have been shown to play significant roles in cell signaling and in aging. For example, two types of enzymes modify normal amino acid residues in signaling proteins. In chemotactic bacteria, the CheR methyltransferase catalyzes the formation of L-glutamyl γ-methyl esters on specific membrane chemoreceptors to control the processing of sensory input [2]. In eucaryotic cells, a distinct enzyme methyl esterifies the α-carboxyl group of the C-terminal leucine residue of the catalytic subunit of protein phosphatase 2A, modulating its interaction with regulatory subunits [3,4]. In both of these cases, specific methylesterases have been described that catalyze the reversal of the methylation reaction and the return to the unmodified residue [5,6]. A third type of enzyme, the isoprenylcysteine methyltransferase, modifies the α-carboxyl group of a lipidated C-terminal cysteine residue on a variety of eucaryotic signaling proteins, but this reaction does not appear to be readily reversible in vivo [7,8]. The last type of enzyme, the L-isoaspartyl/D-aspartyl protein methyltransferase, recognizes spontaneously age-damaged proteins as the first step of a protein repair reaction and is widely distributed in eucaryotic and procaryotic cells [9].

Evidence has been presented for several additional types of protein carboxyl methylation reactions but the enzymes that catalyze these reactions have not yet been identified. For example, the α-carboxyl group of the C-terminal lysine residue of eucaryotic elongation factor 1A has been shown to be methylated in yeast [10]. The Rab3D small GTPase involved in regulated exocytosis has been reported to be modified by an enzyme that is inhibited by *N*-acetyl-*S*-geranylgeranyl-L-cysteine but not *N*-acetyl-*S*-farnesyl-L-cysteine, a known inhibitor of the isoprenylcysteine carboxyl methyltransferase [11]. The β subunit of the xENaC protein component of the sodium channel complex is carboxyl methylated in response to aldosterone in a reaction that has not been chemically characterized [12].

As part of a study on the effect of nucleotides on the L-isoaspartyl/D-aspartyl methyltransferase, tyrphostin A25 was found to stimulate the in vitro methylation of several polypeptides in rat kidney cytosol [13]. Tyrphostins are a class of synthetic low molecular weight antiproliferative compounds that act as protein tyrosine kinase blockers, and the A25 compound is especially active against the EGF receptor kinase [14,15]. It was hypothesized that tyrphostin A25 acts by stabilizing the methyl ester groups on the substrates of the isoaspartyl methyltransferase [13]. Here, we show that tyrphostin A25 addition to a mouse kidney soluble extract greatly stimulates methylation of a distinct set of polypeptides but only in the presence of the protein phosphatase inhibitor vanadate. However, we demonstrate that tyrphostin does not affect the methylation of substrates of the isoaspartyl methyltransferase. In addition, tyrphostin and vanadate do not affect arginine,

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Abbreviations: DMSO, dimethyl sulfoxide; AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]-L-methionine

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lysine, histidine or C-terminal carboxyl methylation. We present evidence that these agents stimulate the methyl esterification of polypeptides via what appears to be a novel modification pathway.

2. Materials and methods

2.1. Chemicals

A stock solution of vanadate was prepared from sodium orthovanadate (Calbiochem, #567540) by suspending the solid in water to a final concentration of 1 mM, adjusting the pH to 10 with NaOH, and heating at 100 °C until the solution clarified. Genistein (#13157-011) and tyrphostin A25 (#13159-017) were from Gibco. Tyrphostins A1, A8, A9, A23, A25, A47, AG1288, B42, B44, B46, B48, B50, and B56 were obtained from Calbiochem (La Jolla, CA). Microcystin-LR (#475815) was obtained from Calbiochem. Genistein, microcystin, and tyrphostins were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mM.

2.2. Tissue fractionation

Pcmt1+/+ and *Pcmt1*−/− mice with a normal or disrupted gene for the L-isoaspartyl/D-aspartyl methyltransferase were generated in a strain background that is about 50% 129svJae and 50% C57BL/6 [16]. After weaning at 20 d, mice were maintained on a NIH-31 Modified Mouse/Rat Diet #7013 on a 12 h cycle of dark and light. Kidneys, liver, heart, brain and testes were removed and added to 8 volumes of 250 mM sucrose, 5 mM HEPES buffer (adjusted to pH 7.2 with Tris base) at 0 °C. Tissues were minced and homogenized with a tight-fitting teflon pestle rotating at 310 rpm for 60 s. Lysates were centrifuged at $13\,000 \times g$ for 50 min and the supernatant (at about 10 mg protein/ml) taken for further analysis. Protein concentrations were determined after precipitation with 10% trichloroacetic acid by the Lowry method using a standard of bovine serum albumin.

2.3. Methylation reactions and gel electrophoresis

Tissue lysate supernatants (60 μg protein) were incubated for 60 min at 37 °C with 3 μl of 12 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham Pharmacia Biotech, 70–81 Ci/mmol, in dilute HCl:ethanol (9:1), pH 2–2.5) in 25 mM HEPES buffer (adjusted to pH 7.5 with Tris base) in a final volume of 30 μl. Sodium vanadate was added when indicated to a final concentration of 100 μM; tyrphostins, genistein, and microcystin (all in DMSO) were added when indicated to final concentrations of 100 μM. Reactions were stopped by adding 30 μl of SDS gel sample buffer (180 mM Tris/HCl, pH 6.8, 4% SDS, 0.1% β-mercaptoethanol, 20% glycerol, and 0.002% bromphenol blue) and heating at 100 °C for 5 min. Samples were electrophoresed at 35 mA for 5 h using either Lammeli [17] or pH 2.4 [18] buffer systems on a gel prepared with 12.6% acrylamide and 0.43% *N*,*N*-methylene-bisacrylamide (1.5 mm thick, 10.5 cm resolving gel, 2 cm stacking gel). Gels were stained with Coomassie Brilliant Blue R-250 for 30 min and destained in 10% methanol, 5% acetic acid overnight. For fluorography, gels were treated with EN^{3}HANCE (Perkin–Elmer Life Sciences). Gels were dried at 70 °C in vacuo and exposed to Kodak X-Omat AR scientific imaging film at −80 °C.

2.4. Amino acid analysis of methylation reactions

Protein precipitates of reaction mixtures incubated in 12.5% trichloroacetic acid, or the portion of a stained SDS gel lane corresponding to the 15-kDa region diced into small pieces, were placed in a 6×50-mm glass vial with 100 μl of 6 N HCl. Acid hydrolysis was then carried out in a Waters Pico-Tag vapor-phase apparatus in vacuo for 20 h at 110 °C. Residual acid was removed by vacuum centrifugation and the resulting hydrolyzed material was resuspended in 50 μl of water and mixed with 1.0 μmol of each of the standards ω-N^G-monomethylarginine (Sigma product M7033; acetate salt) asymmetric ω-N^G, N^G-dimethylarginine (Sigma product D4268; hydrochloride) and $N^\varepsilon, N^\varepsilon, N^\varepsilon$-trimethyllysine (Sigma product T1660) for amino acid analysis by column chromatography. Citrate dilution buffer (500 μl; 0.2 M Na^+, pH 2.2) was added to the hydrolyzed samples before loading onto a cation-exchange column (Beckman AA-15 sulfonated polystyrene beads; 0.9-cm inner diameter × 7-cm column height) equilibrated and eluted with sodium citrate buffer (0.35 M Na^+, pH 5.27) at 1 ml/min at 55 °C. One min fractions were collected and 200 μl aliquots of every other fraction were added to 400 μl of water in 5 ml of scintillation fluor (Safety Solve; Research Products International) and counted. The standards were analyzed using a ninhydrin assay [19]. Under our conditions, the approximate elution times were 37 min for mono, di, and trimethyllysine, 45 min for 1- and 3-methylhistidine, 50 min for methylamine, 65 min for asymmetric dimethylarginine, 70 min for symmetric dimethylarginine, and 76 min for monomethylarginine [19].

2.5. Chemical analyses of protein [^{3}H]methyl group linkages

After gel electrophoresis of a methylation reaction as described above, the 15-kDa region of a lane was sliced out of the wet stained gel and placed in the bottom of a 1.5 ml microcentrifuge tube as previously described [20]. Either 0.2 ml of 2 M Na_2CO_3 or 0.2 ml of 2 M NaOH was added to the tube, which was then placed in a 20 ml scintillation vial containing 5 ml of fluor (Safety Solve) and incubated at 37 °C for 24 h to allow any [^{3}H]methanol formed by alkaline hydrolysis of methyl esters or [^{3}H]methyl amines formed by the alkaline breakdown of methylated arginine residues to diffuse into the scintillation fluid. The vials were then counted. To determine the total radioactivity in the sample, the vials were shaken to mix the complete contents of the microcentrifuge tube with the scintillation fluor and counted again.

Gel slices containing the 15-kDa region were also analyzed for [^{3}H]methanol formation after digestion with proteolytic enzymes as previously described [10]. Briefly, gel slices were immersed in 250 μl of either 1 μg/ml trypsin (Sigma, Type IX, bovine pancreas), 1 μg/ml chymotrypsin (Sigma, type II, bovine pancreas), or 50 μg/ml carboxypeptidase Y (Sigma, from *Saccharomyces cerevisiae*) in a solution of 0.2 M Tris, 0.1 M citrate, pH 6.0, and 1% Triton X-100 in a 1.5 ml microcentrifuge tube. The microcentrifuge tube was placed in a 20 ml scintillation vial containing 5 ml fluor as described above and incubated for 24 h at 37 °C for trypsin or at 30 °C for chymotrypsin and carboxypeptidase Y. The vials were then counted for radioactivity that diffused into the scintillation fluor.

3. Results and discussion

It has been previously shown that addition of a tyrphostin tyrosine kinase inhibitor to rat kidney cytosolic extracts in the presence of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine ([^{3}H]AdoMet) causes a large increase in the [^{3}H]methylation of several species fractionated on SDS gels [13]. We confirmed this observation in mouse kidney soluble extracts with tyrphostin A25 and demonstrated that its stimulatory effect requires the presence of the protein phosphatase inhibitor vanadate (Fig. 1). The effect appears to be at least partially specific, because the tyrosine kinase inhibitor genistein, in the presence or absence of vanadate, has no effect. We show that species migrating as polypeptides of 64 and 42 kDa are more highly methylated and that species of 40, 36, 31, and 15 kDa are apparently methylated de novo in the presence of tyrphostin A25 and vanadate (Fig. 1). The most dramatic change is in the methylation of the 15-kDa species. We also observed an inhibition with tyrphostin and vanadate of the methylation of 20- and 34-kDa species, perhaps as a result of competition for [^{3}H]AdoMet. When DMSO, the solvent for tyrphostin and genistein, was added by itself, the only change observed was the loss of methylation of a minor 23-kDa protein (Fig. 1).

It was initially hypothesized that tyrphostin stabilizes the methylated substrates of the L-isoaspartyl/D-aspartyl methyltransferase [13]. To test this hypothesis, we performed two experiments. In the first place, we tested the effect of tyrphostin and vanadate, alone and in combination, on the rate of demethylation of [^{3}H]methylated species from the protein fraction of tyrphostin and vanadate-treated kidney extracts. We found no significant demethylation up to 180 min of incuba-

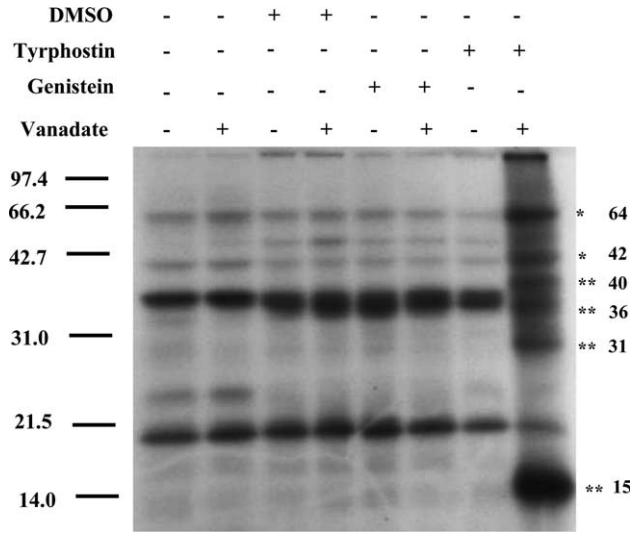

Fig. 1. Tyrphostin A25 and vanadate stimulate the [^{3}H]AdoMet-dependent methylation of polypeptides in mouse kidney soluble extracts. Methylation reactions were performed as described in Section 2 in the presence or absence of 10% DMSO, 100 μM sodium vanadate, 100 μM genistein/10% DMSO, and 100 μM tyrphostin A25/10% DMSO. Polypeptides were fractionated by SDS gel electrophoresis as described in Section 2 and radiolabeled species visualized after gels were exposed to film for 2 months. The position of marker proteins (Bio-Rad low molecular weight standards) electrophoresed in a parallel lane is shown by the lines on the left (rabbit muscle phosphorylase, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg lysozyme, 14.0 kDa). Polypeptides whose methylation is increased upon the addition of tyrphostin and vanadate are indicated by an asterisk on the right of the gel, while polypeptides whose methylation is nearly completely dependent upon tyrphostin and vanadate addition are marked by a double asterisk.

tion under any of the conditions (data not shown), suggesting that the effect of these compounds was not dependent upon the inhibition of a demethylase or direct binding and stabilization of the protein methyl groups. In the second place, we analyzed methylation in extracts of kidneys from wild type mice and mice that had the gene encoding the L-isoaspartyl/D-aspartyl methyltransferase disrupted [16]. The kidneys of each mouse were homogenized and the soluble extracts were incubated with [^{3}H]AdoMet in the presence or absence of vanadate and tyrphostin A25. Reaction mixtures were then analyzed by electrophoresis on either a Lammeli SDS gel at pH 8.5 (Fig. 2A) or on a pH 2.4 gel designed to preserve labile methyl ester linkages (Fig. 2B). We observe the same changes in methylation upon addition of tyrphostin and vanadate with kidney extracts lacking the L-isoaspartyl/D-aspartyl methyltransferase when fractionated on either the Lammeli or pH 2.4 gel system. These results suggest that this enzyme, although responsible for the methylation of a wide variety of proteins in eucaryotic cells, is not involved in the tyrphostin and vanadate-stimulated methylation of the 64-, 42-, 40-, 36-, 31- or 15-kDa species.

In light of the possible co-migration of RNA species with polypeptides on SDS gels [21], we then asked if the species whose methylation is stimulated with tyrphostin and vanadate are indeed polypeptides. When RNaseA was added to reaction mixtures, we found no change in the methylation pattern (Fig. 3). However, when proteinase K was added, all of the methylated species disappeared from the gel (Fig. 3). These results confirm that the tyrphostin and vanadate-stimulated reactions involve protein methylation.

Another major class of eucaryotic methylation reactions involves the modification of the nitrogen atoms in the basic amino acid residues histidine, lysine and arginine [1]. To ask whether the tyrphostin and vanadate-stimulated reactions occurred at these sites, kidney soluble extracts were incubated with [^{3}H]AdoMet and the acid hydrolysis products of the protein fraction were analyzed by high-resolution cation-exchange chromatography (Fig. 4). We observed no stimulation in radiolabeled peaks corresponding to the expected elution positions of mono-, di-, and trimethyllysine, 1- and 3-methylhistidine, N^G-monomethylarginine, N^G,N^G-dimethylarginine, or $N^G,N^{G'}$-dimethylarginine. In fact, we observed decreased methylation in several of these peaks (Fig. 4). We also directly analyzed the major 15-kDa methylated species

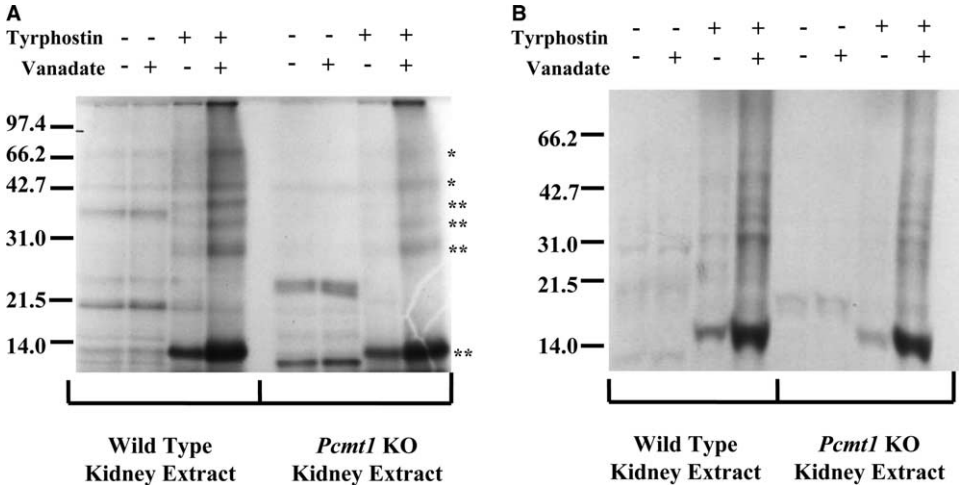

Fig. 2. Protein methylation stimulated by tyrphostin A25 and vanadate is not dependent upon the activity of the L-isoaspartyl/D-aspartyl methyltransferase. Extracts were prepared from kidney of either *Pcmt1*+/+ wild type mice containing the enzyme or *Pcmt1*−/− knockout mice lacking enzyme activity. Methylation reactions were performed in the presence or absence of tyrphostin and vanadate as described in the legend to Fig. 1 and analyzed either with a Lammeli gel (panel A on left) or a pH 2.4 gel (panel B on right) as described in Section 2. Gels were exposed to film for 2 months. The position of marker proteins is given on the left side of each panel and asterisks are used to identify stimulated proteins as in Fig. 1.

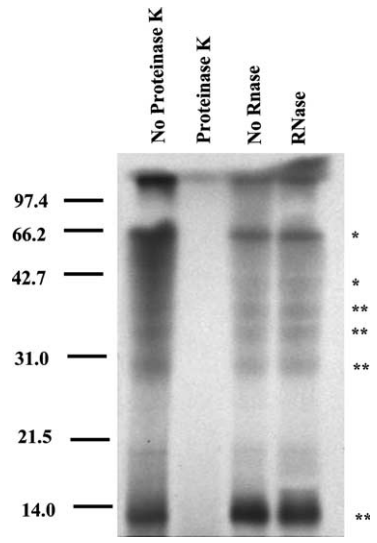

Fig. 3. Tyrphostin and vanadate stimulate protein and not RNA methylation reactions. Methylation of kidney extracts was done as described in Section 2 in the presence of 100 μM tyrphostin A25 and 100 μM sodium vanadate. Either 5 μg of RNaseA (bovine pancreas, Sigma #R6513) or 10 μg of proteinase K (*Tritirachium album*, Sigma #P2308) was added to the reaction mixture and the incubation continued for 30 min at room temperature for the RNaseA or overnight for proteinase K. Reactions were then analyzed as described in Fig. 1. The gel was exposed to film for 23 days. The position of molecular weight markers is shown on the left with horizontal lines and methylated polypeptides are identified by asterisks on the right as in Fig. 1.

purified from SDS gel electrophoresis experiments by chromatography as shown in Fig. 4. Here, we found no radioactivity in any of the lysine, histidine, or arginine derivatives (data not shown). Additionally, we found no radioactivity in the 50 min position of methylamine, the expected hydrolysis product of side chain glutamine methylation.

The results described above suggested that tyrphostin and vanadate might stimulate a protein carboxyl methylation reaction distinct from that catalyzed by the L-isoaspartyl/D-aspartyl methyltransferase. This type of modification can be identified by treating [^{3}H]methylated proteins with base and analyzing for the presence of the volatile [^{3}H]methanol product. Here, we treated gel slices containing the 15-kDa [^{3}H]methylated protein from kidney extracts with either Na_2CO_3 or NaOH. Upon treatment with NaOH, we found that at least 62% of the total radioactivity was volatile and could be accounted for as [^{3}H]methanol. When the gel slice was treated with Na_2CO_3, at least 80% of the total radioactivity was volatile (data not shown). These results suggest that the tyrphostin and vanadate-stimulated methylation involves the formation of methyl ester linkages.

To determine whether this methylation may be occurring at the C-terminal alpha-carboxyl group, we treated the labeled 15-kDa species with enzymes that can cleave a methyl ester linkage at this position [10]. However, we found no production of volatile radioactivity as [^{3}H]methanol upon treatment with chymotrypsin, trypsin, and carboxypeptidase Y (data not shown). These enzymes would be expected to liberate [^{3}H]methanol from the methylated C-terminal leucine residue of protein phosphatase 2A [10], the methylated lysine residue of elongation factor 1A [10], and isoprenylated cysteine residues of various species [22], respectively. We also chromatographed the carboxypeptidase Y digestion products on cation exchange chromatography as described [23] and found no evidence for the presence of either aspartate or glutamate side chain methyl esterification (data not shown). These results suggest that the tyrphostin and vanadate-stimulated methylation is a novel type of protein carboxyl methylation reaction.

We next tested to see if tyrphostin A25 and vanadate could stimulate methylation in other mouse tissues. Heart, brain, and testis lysates were incubated with [^{3}H]AdoMet in the presence or absence of vanadate and tyrphostin (Fig. 5). In heart extracts, tyrphostin with or without vanadate stimulated the methylation of 64-, 42-, 40-, 36-, 31-, 18- and 15-kDa polypeptides. Except for the 18-kDa species, methylation of all of these polypeptides were also observed to be stimulated in kidney extracts. In brain extracts, we observed only a slight stimulation of a 24.4-kDa polypeptide with tyrphostin, and the combination of tyrphostin and vanadate actually caused a general inhibition of the basal methylation levels. Finally, in testis, there was little change in the methylation pattern in the presence or absence of tyrphostin and vanadate. These results

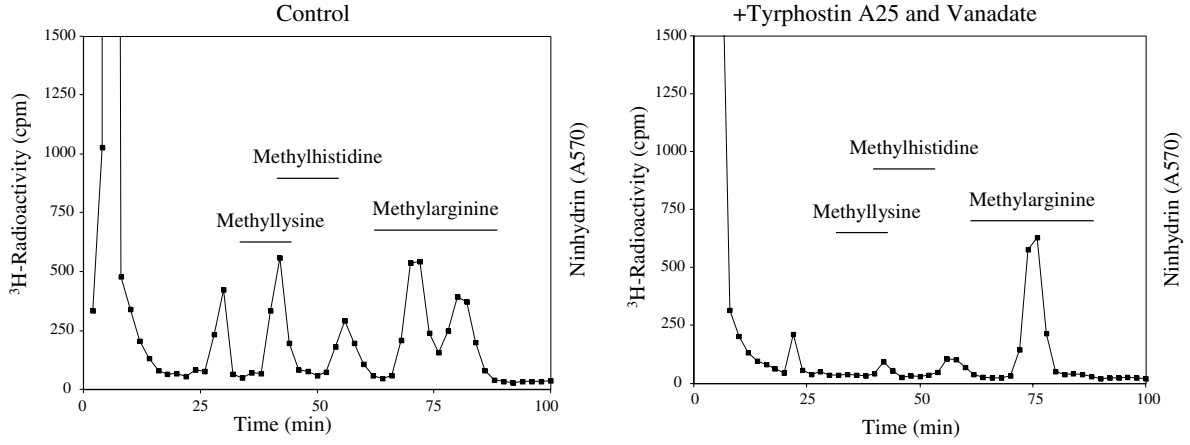

Fig. 4. Amino acid analysis of ^{3}H-methylated proteins in mouse kidney extracts. Methylation of soluble tissue extracts was performed in the presence or absence of 100 μM vanadate and 100 μM tyrphostin A25/10% DMSO as described in Fig. 1. After precipitation with trichloroacetic acid, proteins were acid hydrolyzed and chromatographed by high resolution cation exchange chromatography as described in Section 2.

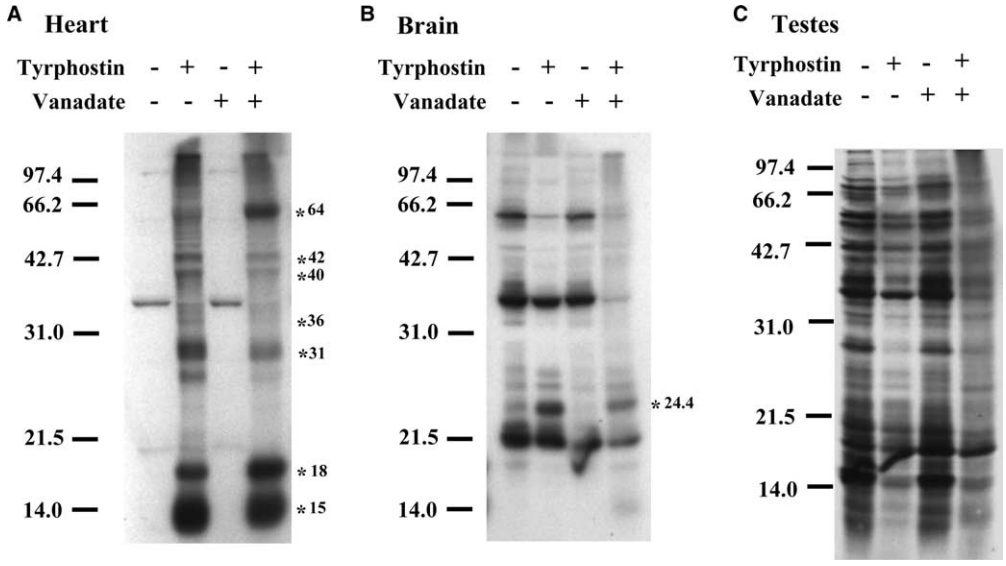

Fig. 5. Tyrphostin A25 and vanadate affect methylation of polypeptides in extracts of mouse heart, brain, and testis. Methylation of soluble tissue extracts was performed in the presence or absence of 100 μM vanadate and 100 μM tyrphostin A25/10% DMSO as described in Fig. 1. Gels were exposed to film for 33 days. The position of molecular weight markers is shown with horizontal lines and of that of the stimulated methylated polypeptides by asterisks.

suggest that the tyrphostin/vanadate stimulated methylation system is active in both kidney and heart with the increased methylation of at least some similarly sized polypeptides. On the other hand, this pathway does not appear to operate in testes and may be limited to a 24.4 kDa species in brain. We also tested to see if tyrphostin and vanadate had an effect on methylation of extracts of the yeast *Saccharomyces cerevisiae*. Neither tyrphostin nor vanadate had any effect when added alone, and when both compounds were added to the reaction we saw a decrease in methylation (data not shown).

We then asked if the increase in methylation seen by the addition of tyrphostin was specific to tyrphostin A25 or if other tyrphostins also had the same effect on methylation (Table 1). Tyrphostins A1, A8, A9, A23, A25, A47, AG1288, B42, B44, B46, B48, B50, and B56 [14,15] were incubated with kidney cytosol with and without vanadate along with [^{3}H]AdoMet. With the exception of tyrphostin A47, we found that none of the other tyrphostins had any effect on the methylation of the endogenous kidney cytosolic proteins. Since many of these tyrphostin species inhibit the epidermal growth factor receptor kinase activity nearly equally as well as A25 (Table 1) and because many of the inactive compounds are either broad spectrum inhibitors of protein tyrosine kinases [24–26] or are active on other types of these enzymes [27,28], the stimulation of methylation with A25 and A47 may be due to its interaction with a distinct type of protein. Alternatively, A25 and A47 may be specific for a yet undescribed class of protein tyrosine kinases, or perhaps a distinct class of molecules.

Table 1
The effect of tyrphostin A25 derivatives on the methylation of kidney cytosolic proteins[a]

Tyrphostin	AG#	IC_{50} (μM)[b]	Effect on stimulation of methylation
A1	AG 9	>1250	–
A8	AG 10	560	–
A9	AG 17	460	–
A23	AG 18	35	–
A25	AG 82	3	+++
A47	AG 213	2.4	++
	AG 1288		–
B42	AG 490	2	–
B44	AG 527	0.4	–
B46	AG 555	0.7	–
B48	AG 494	0.7	–
B50	AG 835	0.86	–
B56	AG 556	1.1	–

[a] Methylation of kidney extracts was performed with 100 μM of the indicated tyrphostin and 100 μM vanadate as described in Section 2.
[b] IC_{50} values for the inhibition of the phosphorylation of polyGAT by EGFRK [22,23].

Sodium vanadate is a known protein tyrosine phosphatase inhibitor but also has additional effects on a number of other types of hydrolytic enzymes, including the Na^+/K^+ ATPase and small molecule phosphatases [29]. To ask if the effect of vanadate might be mimicked by a distinct type of protein phosphatase inhibitor, we substituted microcystin-LR for vanadate (Fig. 6). We found, however, that it showed no stimulation of methylation in kidney extracts incubated with tyrphostin. This result suggests some specificity to the action of vanadate. It is possible, in fact, that vanadate acts at a site or sites distinct from those of protein phosphatases. Further work will be needed to establish the molecular basis of the vanadate, as well as the tyrphostin, effects.

The nature of the enzyme or enzymes that catalyze the tyrphostin/vanadate-stimulated protein carboxyl methylation observed here is unknown. However, if the effects of tyrphostin and vanadate are due to their interactions with protein kinases and phosphatases, then this system may well represent an additional example where there are linkages between protein phosphorylation reactions involved in cellular signaling and protein methylation reactions. For example, the activity of protein arginine methyltransferase 1 is stimulated by the NGF tyrosine kinase [30] and participates in the signaling cascade of phosphorylated forms of the STAT1 protein [31]. Protein

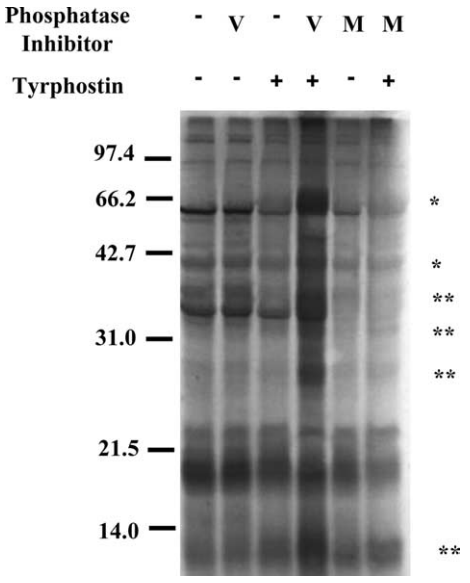

Fig. 6. The effect of microcystin-LR on the methylation of kidney soluble proteins. Methylation of kidney extract proteins was performed in the presence or absence of 100 μM tyrphostin A25/10% DMSO, 100 μM vanadate (V) and 100 μM microcystin-LR/10% DMSO (M) as described in Section 2. The gel was exposed to film for 2 months. The position of molecular weight markers is shown on the left with horizontal lines and of enhanced methylated polypeptides by asterisks on the right as in Fig. 1.

arginine methyltransferase 5 binds specifically to the JAK2 kinase [19,32]. The major protein phosphatase 2A is regulated by reversible carboxyl methylation [3,4,6] and the cheB methylesterase of bacterial chemotaxis is itself regulated by phosphorylation [5]. Several tyrosine kinase inhibitors are presently in clinical trials as anti-tumor drugs [33] and it would be interesting to see if any of these agents also stimulate protein carboxyl methylation reactions. The situation is complex because both the stimulatory effects of tyrphostin and vanadate would seemingly involve both the inhibition and stimulation of protein phosphorylation; multiple enzymes may be involved here, or the sites of action of these compounds may in fact be distinct from enzymes involved in reversible protein phosphorylation reactions.

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