Research Article

Cancer Prevention Research

Proteomic Profiling of Potential Molecular Targets of Methyl-Selenium Compounds in the Transgenic Adenocarcinoma of Mouse Prostate Model

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Abstract

Because the Selenium (Se) and Vitamin E Cancer Prevention Trial (SELECT) failed to show the efficacy of selenomethionine for prostate cancer prevention, there is a critical need to identify safe and efficacious Se forms for future trials. We have recently shown significant preventive benefit of methylseleninic acid (MSeA) and Se-methylselenocysteine (MSeC) in the transgenic adenocarcinoma mouse prostate (TRAMP) model by oral administration. The present work applied iTRAQ proteomic approach to profile protein changes of the TRAMP prostate and to characterize their modulation by MSeA and MSeC to identify their potential molecular targets. Dorsolateral prostates from wild-type mice at 18 weeks of age and TRAMP mice treated with water (control), MSeA, or MSeC (3 mg Se/kg) from 8 to 18 weeks of age were pooled (9-10 mice per group) and subjected to protein extraction, followed by protein denaturation, reduction, and alkylation. After tryptic digestion, the peptides were labeled with iTRAQ reagents, mixed together, and analyzed by two-dimensional liquid chromatography/tandem mass spectrometry. Of 342 proteins identified with >95% confidence, the expression of 75 proteins was significantly different between TRAMP and wild-type mice. MSeA mainly affected proteins related to prostate functional differentiation, androgen receptor signaling, protein (mis)folding, and endoplasmic reticulum–stress responses, whereas MSeC affected proteins involved in phase II detoxification or cytoprotection, and in stromal cells. Although MSeA and MSeC are presumed precursors of methylselenol and were equally effective against the TRAMP model, their distinct affected protein profiles suggest biological differences in their molecular targets outweigh similarities. *Cancer Prev Res; 3(8); 994–1006. ©2010 AACR.*

Introduction

Chemoprevention of prostate carcinogenesis is a plausible and necessary approach to deal with the prostate cancer problem at the root (1). Previous studies have suggested that supplementation of selenium (Se) may modify the risk of and prevent human prostate cancer (2–4). However, the National Cancer Institute stopped the Selenium and Vitamin E Cancer Prevention Trial (SELECT) in October 2008, several years ahead of schedule, because of the failure to show an efficacy of selenomethionine (SeMet) for prostate cancer prevention in North American men (5). Possible reasons for failure to show SeMet efficacy have been reviewed, including dosage, chemical form, and Se status of subjects (6). In hindsight, experiments with preclinical prostate cancer animal models conducted before (7) and since SELECT was initiated including our study with xenograft models (8, 9) did not support any *in vivo* anticancer activity of SeMet.

In contrast, we have shown that orally administered second-generation selenium compounds (in reference to SeMet and selenium inorganic salts) methylseleninic acid (MSeA) and Se-methylselenocysteine (MSeC) inhibit the *in vivo* growth of DU145 and PC-3 human prostate cancer xenograft in athymic nude mice (9). Our group has also reported recently the *in vivo* efficacy of daily oral administration of MSeA and MSeC against primary carcinogenesis in the transgenic adenocarcinoma mouse prostate (TRAMP) model (10). We showed that MSeA given to TRAMP mice from 10 or 16 weeks of age increased their survival to 50 weeks of age and delayed the death due to synaptophysin-positive neuroendocrine (NE) carcinomas and synaptophysin-negative prostate lesions, and seminal vesicle hypertrophy (10). Because of the metabolic and biological differences that have been well documented between SeMet and other Se forms (9, 11), the failure of SeMet in the SELECT study should not be equated to all Se forms as "ineffective" for prostate cancer prevention. We believe that the quest for effective Se agents takes on even greater significance and urgency

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(10) because there is no other clinically proven effective prostate cancer preventive agent other than the controversial 5-α reductase II inhibitor Finasteride that blocks the intraprostatic generation of the active androgen dihydrotestosterone but with significant side effects and questionable survival benefit (12). The chemopreventive efficacy of MSeA and MSeC shown in the preclinical models above and the well-tolerated nature of the doses tested support these second-generation Se compounds as promising candidates for consideration for future translational studies.

In spite of cell culture studies conducted with these Se forms, their *in vivo* molecular targets for chemoprevention in the prostate remain poorly defined. Prostate carcinogenesis, as that in other organs, involves changes of proteins in multiple pathways in different types of cells. Characterizing the effects of various Se forms on the proteome changes may lead to a better understanding of their potential chemopreventive protein targets or mediators *in vivo*.

Differential proteomics using two-dimension liquid chromatography coupled with tandem mass spectrometric analysis (two-dimensional LC-MS/MS) is a powerful tool to investigate the global protein expression changes. Two-dimension liquid chromatography based on different characteristics of peptides such as polarity and charge substantially reduces sample complexity. Modern mass spectrometry allows not only peptide identification but also quantitation. Compared with other approaches such as two-dimensional electrophoresis, two-dimensional LC-MS/MS has the advantage of both sensitivity and convenience. Furthermore, it is easy to be standardized, and thus, the reproducibility can be guaranteed. For the purpose of relative quantitation, several kinds of reagents such as ICAT (13) and ^{18}O (14) are used to introduce labeling tags to the proteins or peptides. Presently, isobaric tag for relative and absolute quantization (iTRAQ, Applied Biosystems) is the most widely used labeling system (15) consisting of four or eight multiplexing reagents to label peptides from different groups (see Fig. 1A for principle). The iTRAQ reagents use a N-hydroxysuccinimide ester derivative to modify primary NH_2 groups in peptides by linking a mass balance group (carbonyl group) and a reporter group (based on N-methylpiperazine) to peptides through the formation of an amide bond. Peptides labeled with these isobaric tags are indistinguishable in the MS survey scan. Fragmentation under collision-induced dissociation generates product ions (-b, -y types) for further peptide identification and enables relative quantitation by comparing peak areas of reporter ions (*m/z* from 114-117). Therefore, identification and quantitation are done concurrently.

Here, we report the use of iTRAQ-based differential proteomics to investigate proteins involved in TRAMP carcinogenesis and how those proteins were modulated by MSeA and MSeC using dorsolateral prostate samples banked from our published study (see experimental setup and analytic flow chart in Fig. 1B; ref. 10). Although MSeA and MSeC are considered precursors (see chemical structure and hypothetical metabolism in Fig. 1C) of the methylselenol metabolite pool, which has been believed to be the active chemopreventive species (11) and they are about equally effective against the TRAMP dorsal lateral prostate lesion progression (10), the observed differences in the affected protein profiles suggest differences in their molecular targets may far outweigh similarities, challenging the currently held paradigm of their cancer chemopreventive activities. To our knowledge, this is the first study that exploits a proteomic approach by two-dimensional LC-MS/MS with iTRAQ labeling for the relative quantitative proteomic profiling of TRAMP prostate carcinogenesis and the effect of chemopreventive methyl Se compounds.

Materials and Methods

Reagents

iTRAQ 4-plex reagent kits were purchased from Applied Biosystems (ABI). Antibody for glutathione *S*-transferase μ (GSTM) was purchased from Abcam; antibodies for calponin-1 and GRP78 were purchased from Santa Cruz. The BCA protein quantitation kit was from Pierce.

TRAMP model and tissue selection

The TRAMP model, originally developed by Dr. N. Greenberg (16), to some extent, mimics the natural history and progression of human prostate cancer (17). The probasin promoter–driven SV40 T-antigen expression abrogates p53 and Rb tumor suppressor proteins to propel prostatic lesion growths. More recent work has shown that the poorly differentiated carcinomas in this model are NE-like carcinomas that are androgen insensitive (no androgen receptor, AR), express synaptophysin, and belong to a distinct lineage from the prostatic intraepithelial neoplasia and well-differentiated and moderately differentiated adenocarcinoma lesions (18). Furthermore, the incidence of these NE carcinomas is mouse strain dependent: in the C57BL/6 background, a lifetime incidence of ~20% NE carcinomas versus in the FVB background, 87% NE carcinoma incidence was recorded by as early as 16 weeks of age (18). In both strains, these NE carcinomas mostly arise in the ventral prostate instead of the dorsal-lateral prostate (DLP; ref. 18). As is understood today, the TRAMP therefore represents at least two models of prostate carcinogenesis: one that approximates the AR/probasin promoter/T-antigen–mediated glandular prostate epithelial cancer formation in the DLP and another that simulates the development of NE carcinomas in the ventral prostate, and is T-antigen driven but independent of AR. The latter is also important for human prostate cancer in that the number of NE cells correlates with the stage, Gleason grades, and survival in castration-recurrent prostate cancer in the clinical cases (19). In addition to these prostate lineages of carcinogenesis, sarcomatoid lesions arising in the seminal vesicles often add to and complicate the overall tumor burden in the older mice of the C57BL/6 strain (20).

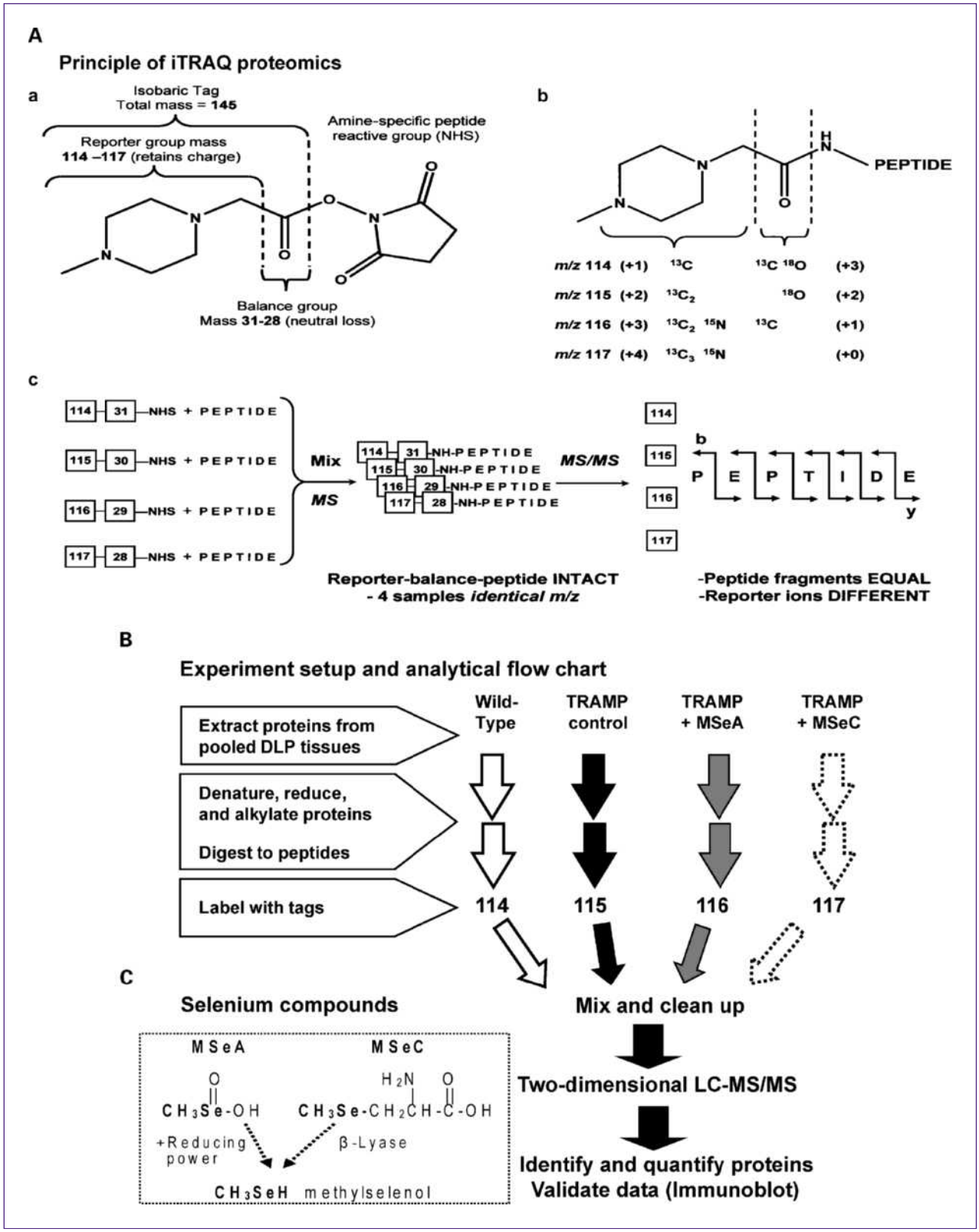

Fig. 1. A, principle of global protein expression analysis by LC-MS/MS coupled with amine-specific isobaric tags (iTRAQ; reproduced with permission of The American Society for Biochemistry and Molecular Biology; ref. 15). B, outline of experimental design and major analytic steps. C, chemical structure of selenium compounds used for treating TRAMP mice and their hypothetical metabolism to methylselenol.

Because of the complex lineages of carcinogenesis discussed above, we focused on the banked frozen DLP tissues from 18-week-old wild-type and TRAMP mice in the C57BL/6 strain from our previous work (10) for the proteomic work. Figure 1B outlines the treatments and iTRAQ labeling scheme. Briefly, male TRAMP mice at 8 weeks of age (n = 10 per group) received a daily oral dose of water (TRAMP control), MSeA (TRAMP MSeA), or MSeC (TRAMP MSeC) delivered to the back of the tongue (five times per week) at the dosage of 3 mg Se per kilogram body weight. At 18 weeks of age, the mice from each group were sacrificed, and the lower genital urinary tract including seminal vesicle, prostate, testes, and bladder was dissected and weighed. The DLP was further dissected on a bed of ice and weighed. Approximately half of each DLP was fixed for routine histopathologic evaluation, and the other portion was snap frozen on dry ice and stored at −70°C. Ten wild-type mice of 18 weeks of age were scarified for the collection of DLP as age-matched noncarcinogenic baseline group (wild-type).

Protein extraction

Because of the NE carcinomas arise early and almost exclusively in the ventral gland, we used only dissectable DLP tissue from each group, thus excluding one mouse with a 2.6 g synaptophysin-positive NE carcinoma in the water-control group (10). Briefly, 9 or 10 DLPs from each groups were pooled, and proteins were extracted into lysis buffer [0.5 mol/L triethylammonium bicarbonate, 0.05% SDS, 0.1% Triton X-100 (pH 8.5)]. Concentration of proteins was determined by the BCA method.

iTRAQ labeling

Proteins were first denatured, reduced, alkylated, and digested to peptides. Briefly, 30 μg of protein from each group in 20 μL lysis buffer were mixed with 2 μL 0.05 mol/L tris-(2-carboxyethyl)phosphine and incubated at 60°C for 1 hour. Then, 1 μL methyl methane thiosulfonate was added to alkylate free cysteine residues. Subsequently, each sample was digested with 10 μg trypsin at 37°C overnight. Peptides were labeled with Applied Biosystems iTRAQ Reagents according to the manufacturer's instruction. Peptides from the wild-type mice were labeled with iTRAQ Reagents 114; and peptides from TRAMP control, MSeA-, and MSeC-treated groups were labeled with 115, 116, and 117, respectively. Labeled peptides were mixed and cleaned up by solid phase extraction with MCX cartridge and then sent to the Mass Spectrometry and Proteomics Facility of the University of Minnesota.

Two-dimensional LC-MS/MS analysis

The mixture of all labeled peptides was first fractioned using strong cation exchange chromatography (SCX). Fifteen fractions were collected and subjected to LC-MS/MS analysis by capillary C18 LC online with a QSTAR pulsar i mass spectrometer. Product ion spectra acquisition method was set up to acquire data on the four most abundant peaks as fast as the instrument could run, in a continuous fashion.

Data processing

Protein identification and relative quantitation were carried out using the ProteinPilotTM 2.0.1 software (Applied Biosystems). Data were searched against the National Center for Biotechnology Information database (version 2006-12-15). Precursor peptide mass and product fragment ion mass tolerances were dynamically adjusted by the software. As fixed modifications, the software allows one missed cleavage of trypsin, MMTS-labeled cysteines, and oxidized methionine. Only proteins identified with at least 95% confidence are reported (21).

The peak areas of the "reporter" ions (m/z 114, 115, 116, and 117) were used for peptide/protein abundance quantitation. The relative peptide abundance in wild-type prostate compared with that of TRAMP mice was expressed as the ratio of the peak area at m/z 114 to the peak area of m/z 115. Similarly, the relative peptide abundance in MSeA- and MSeC-treated TRAMP prostate compared with that of the water-treated control TRAMP mice was expressed as the ratio of the peak area at m/z 116 and 117 to the peak area of m/z 115, respectively. Peptides shared among proteins were not used in quantitation. Relative abundance of each protein was expressed as the average iTRAQ ratios of all informative peptides from the given protein. The P value and error factor for each protein were calculated by the software (21). To normalize differences in protein loading across the samples, all observed protein ratios were divided by the average iTRAQ ratio for that experiment. Only proteins identified with a minimum of two peptides, quantization results with $P < 0.05$, and error factor of <2 were considered a positive call. The final results obtained from the ProteinPilotTM 2.0.1 software were exported to Microsoft Excel for further analyses.

Ingenuity Pathway Analysis, a literature-based software, was used to classify the pathways in which the identified proteins are involved.

Western blot analyses

Protein extraction and quantization were described as for the LC-MS/MS study. Western blots were performed as described by Jiang et al. (22); the signals were detected by enhanced chemofluorescence with a Storm 840 scanner (Molecular Dynamics). Dilutions of antibodies were as follows: GSTM, 1:3,000; Calponin-1 and GRP78, 1:200.

Results

Orally administrated MSeA and MSeC exerted similar inhibitory efficacy on TRAMP-driven DLP growth

As a surrogate semiquantitative indicator of prostate epithelial carcinogenesis, the DLP weight of the TRAMP mice at 18 weeks (101 mg, n = 9 mice, excluding 1 mouse with a 2.6-g synaptophysin-positive NE carcinoma) was

2.37-fold greater than that of the age-matched wild-type mice (30 mg, $n = 10$; $P < 0.001$). The DLP weight of the TRAMP mice treated with MSeA or MSeC for 10 weeks (from 8-18 wk of age) was significantly less than the water-treated TRAMP mice, being 62 and 58 mg, respectively ($P < 0.001$ for each compound). These data have been reported previously (10). Histologic evaluation of the DLP sections supported retarded lesion progression with more mice displaying lower grade lesions such as low-grade prostatic intraepithelial neoplasia in the MSeA- or MSeC-treated groups than in the water-treated group (10).

iTRAQ proteomics identified GSTM1, calponin-1, and GRP78 as suppressed proteins in TRAMP prostate with differential attenuations by MSeC versus MSeA

Figure 2 shows the identification of GSTM1, using the MS spectrum of double-protonated peptide ADIVENQVMDTR (*m/z* 767.8862; A) and the *m/z* of the product b- and y-ion series (B). Together with other peptides identified from this protein, 50% of the amino acid sequence was covered (C).

The peak areas of iTRAQ reporter ion at *m/z* 114, 115, 116, and 117 were used to measure the relative amount of GSTM1 in DLP of wild-type mice, and TRAMP mice treated with water (set as 1), MSeA, or MSeC. Altogether, 12 peptide fragments from GSTM1 were used for quantitation (Fig. 2D). Statistical analysis indicated that GSTM1 was significantly lower in the TRAMP DLP compared with wild-type DLP (37% decrease). MSeA treatment did not attenuate this decrease of GSTM1 abundance, whereas MSeC treatment completely reversed or even overcorrected the TRAMP-induced decrease of GSTM1 abundance.

Immunoblot analyses (Fig. 3A, image and normalized densitometric ratio) confirmed the GSTM1 response

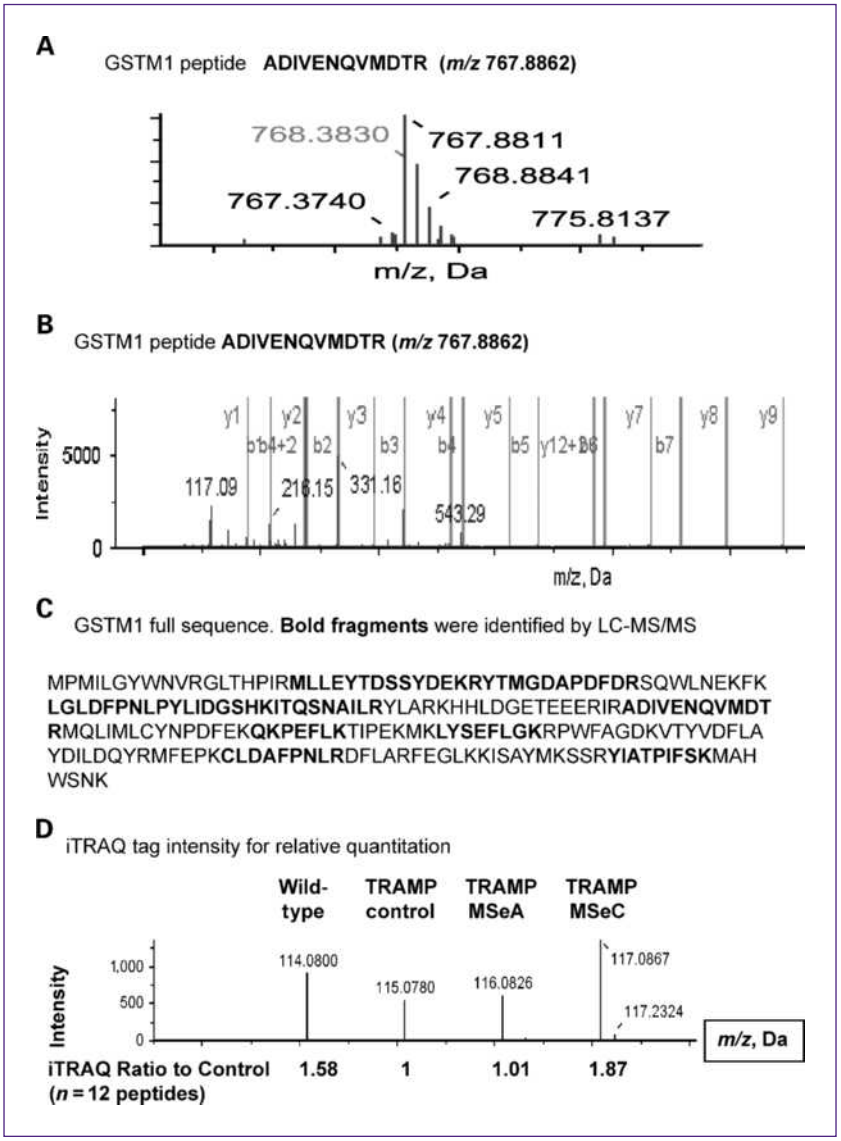

Fig. 2. Identification and quantitation of GSTM1 by iTRAQ proteomics. Precursor ions (A) and product ions (B) of the MS and MS/MS spectra of double-protonated peptide ADIVENQVMDTR (*m/z* 767.8862). C, amino acid sequence of GSTM1. Bold regions, the peptide sequences identified. D, graphic distribution of tag intensities for one GSTM1 peptide and the average tag ratios of all identified GSTM1 peptides.

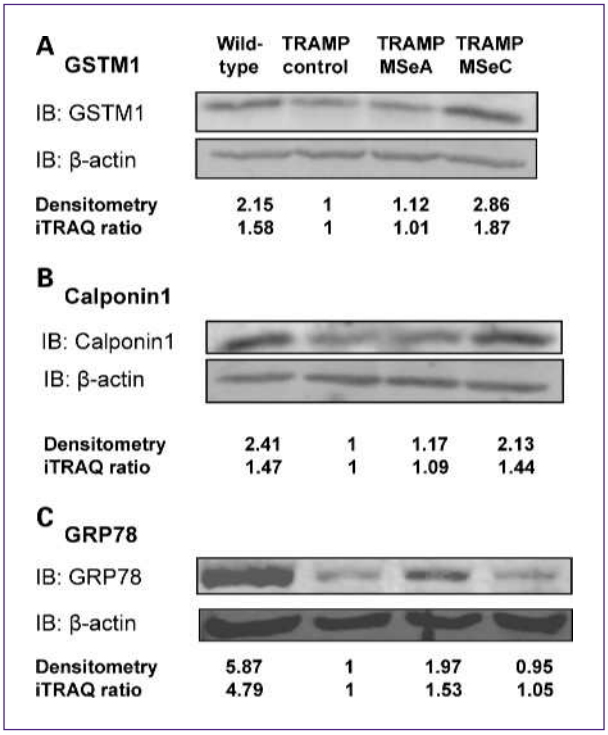

Fig. 3. Immunoblot (IB) analyses of (A) GSTM1, (B) Calponin-1, and (C) GRP78 to validate protein quantitation by iTRAQ proteomics. The images show immunoblots of the pooled tissue lysates from different groups and densitometric quantitation normalized to β-actin loading.

patterns above. Similarly, we validated the expression patterns of Calponin-1, which showed a lack of influence by MSeA but a complete reversal by MSeC of TRAMP-associated decreased expression (Fig. 3B) and GRP78, which showed a partial restoration by MSeA, but not by MSeC (Fig. 3C). The excellent agreement between these two methodologies suggests the iTRAQ proteomic approach is a reliable way to determine the relative abundance of proteins from different groups in our model.

Cutoff threshold values for statistically significant protein expression changes by iTRAQ

Before subjecting the identified proteins to further bioinformatic analyses, it was necessary to set threshold cutoff values for statistically acceptable significant changes of protein abundance. To experimentally approach this, we compared the random variations in the iTRAQ detection by self-pairing of tag-114 and tag-116 using a ventral prostate protein extract sample (Fig. 4). The tag-114 and tag-116 intensity data for all identified peptides showed a correlation slope of close to unity (Fig. 4A). When two or more peptides were available for the identification of a given protein, the average self-pair ratio scattered within 20% of unity (Fig. 4B). Additional self-pairing between tag-114 and tag-115 led to the same results as shown in Fig. 4B (data not shown). These data were in excellent agreement with those of Kassie et al. (23), showing ±20% range encompassing the random variations of self-paired lung tissue samples. Therefore, we consider tag ratios within 0.8 to 1.2 not different in protein abundance from that of the reference group.

Protein changes related to TRAMP carcinogenesis

In total, we identified 424 proteins expressed in the DLP of 18-week-old TRAMP mice and wild-type mice by two-dimensional LC-MS/MS. Of which, 342 were identified with >95% confidence as specified in the Materials and Methods section. Based on the cutoff threshold for significant difference established above, i.e., expression ratio <0.8 or >1.2 against that of the TRAMP control set as 1, the expression levels of 75 of the 342 proteins were different between wild-type DLP and TRAMP DLP (Table 1A and B).

Of these 75 proteins, 33 (44%) proteins were increased in TRAMP DLP (Table 1A), and 42 (56%) proteins

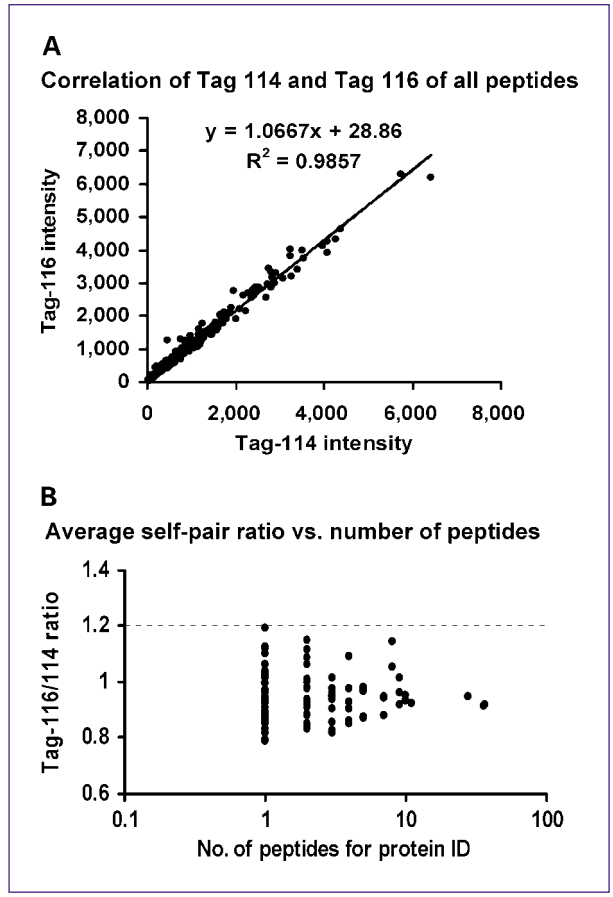

Fig. 4. Self-pairing of tag 114 and 116 for a prostate protein extract sample to determine the cutoff threshold. A, correlation of tag 114 and tag 116 intensities of all the peptides identified with >95% confidence (n = 678 peptides). Solid line, expected perfect equal labeling by the two tags of the same peptides (slope = 1). B, the distribution of the average ratio of tag 114 and tag 116 intensities of proteins as a function of the number of peptides used for protein identification and quantitation (n = 123 proteins). Unity ratio (1) is expected for self pairing.

Table 1. Proteins that are either significantly upregulated or downregulated in TRAMP DLP and their modulation by MSeA or MSeC

Entry no.	Access no.	Protein	iTRAQ abundance ratio			Possible Function in cancer
			wild: TRAMP	MSeA: TRAMP	MSeC: TRAMP	
A. Upregulated proteins in TRAMP DLP						
1	gil94381071	***Deleted in malignant brain tumors 1 isoform c****	0.35	***1.30***	***1.72***	
2	gil31980848	***Seminal vesicle protein, secretion 2***	0.56	***0.60***	***1.32***	
3	gil312588	***Biliary glycoprotein***	0.43	***0.65***	***0.64***	
4	gil6753096	***Apolipoprotein A-I***	0.61	***0.65***	***0.71***	
5	gil7304889	**Annexin A4**†	0.78	**1.24**	NS‡	
6	gil45598372	**Brain abundant, membrane-attached signal protein 1**	0.75	**0.78**	NS	
7	gil6681015	**Cysteine-rich protein 1**	0.75	**0.69**	NS	
8	gil23956086	**Hemopexin**	0.75	**0.52**	NS	
9	gil7305531	**Seminal vesicle secretion 6**	0.75	**0.34**	NS	
10	gil116089316	*Hypothetical protein LOC71395*§	0.39	NS	*1.69*	
11	gil6755212	*Proteasome (prosome, macropain) 28 subunit, α*	0.37	NS	*0.76*	
12	gil34328185	*Prosaposin*	0.46	NS	*0.59*	Oncogene
13	gil123794006	*FUSE-binding protein 2*	0.54	NS	*0.78*	
14	gil84875537	*Nucleolin*	0.57	NS	*0.77*	
15	gil40254525	*Tropomyosin 3, gamma*	0.77	NS	*0.78*	
16	gil94383594	High mobility group box 2	0.30	NS	NS	Oncogene
17	gil6677991	Solute carrier family 12, member 2	0.31	NS	NS	
18	gil21359820	Myoglobin	0.40	NS	NS	
19	gil6754508	LIM and SH3 protein 1	0.43	NS	NS	
20	gil6754570	Annexin A1	0.46	NS	NS	
21	gil68131562	Quiescin Q6 isoform a	0.49	NS	NS	
22	gil31981822	Cystatin C	0.49	NS	NS	
23	gil6671762	Creatine kinase, muscle	0.53	NS	NS	
24	gil13195646	LIM only protein HLP	0.64	NS	NS	
25	gil6678359	Transketolase	0.64	NS	NS	
26	gil83745120	Ribosomal protein, large P2	0.69	NS	NS	
27	gil15029896	Hnrpd protein	0.70	NS	NS	
28	gil27754007	NADH dehydrogenase (ubiquinone) 1, α/β subcomplex, 1	0.70	NS	NS	
29	gil6755911	Thioredoxin 1	0.71	NS	NS	
30	gil7949094	Nucleobindin 2	0.72	NS	NS	
31	gil61743961	AHNAK nucleoprotein isoform 1	0.73	NS	NS	
32	gil6754976	Peroxiredoxin 1	0.74	NS	NS	
33	gil6678413	Triosephosphate isomerase 1	0.78	NS	NS	
B. Downregulated proteins in TRAMP DLP						
34	gil6671664	Calnexin	1.22	NS	NS	
35	gil6679465	protein kinase C substrate 80K-H	1.76	NS	NS	
36	gil31981909	Aldo-keto reductase family 1, member B3 (aldose reductase)	2.97	NS	NS	
37	gil18857865	ERp44	3.12	NS	NS	
38	gil6679158	*Nucleobindin 1*	2.50	NS	*0.72*	
39	gil94717635	*Progesterone receptor membrane component*	1.21	NS	*1.60*	

(*Continued on the following page*)

Table 1. Proteins that are either significantly upregulated or downregulated in TRAMP DLP and their modulation by MSeA or MSeC (Cont'd)

Entry no.	Access no.	Protein	iTRAQ abundance ratio			Possible Function in cancer
			wild: TRAMP	MSeA: TRAMP	MSeC: TRAMP	
40	gil21704156	*Caldesmon 1*	1.26	NS	*1.29*	
41	gil31980648	*ATP synthase, H+ transporting mitochondrial F1 complex β*	1.33	NS	*1.31*	
42	gil10946574	*Creatine kinase, brain*	1.36	NS	*1.33*	
43	gil94367355	*Similar to 60S ribosomal protein L5*	1.41	NS	*1.29*	
44	gil31981520	*Pancreatic ribonuclease*	1.43	NS	*1.21*	
45	gil584951	*Calponin-1 (Calponin H1, smooth muscle)*	1.47	NS	*1.44*	
46	gil6754084	*Glutathione S-transferase, mu 1*	1.58	NS	*1.87*	Suppressor
47	gil6680121	*Glutathione S-transferase, μ 2*	1.65	NS	*1.45*	Suppressor
48	gil31981302	*Annexin A6*	1.64	NS	*1.45*	
49	gil6678740	*Lumican*	1.71	NS	*1.62*	
50	gil6755714	*Transgelin*	1.93	NS	*1.50*	Suppressor
51	gil94403723	*Similar to Heat shock 70-kDa protein 1B (HSP70.1)*	2.26	NS	*1.44*	
52	gil89574165	**Succinate dehydrogenase complex subunit A**	1.25	**0.76**	NS	
53	gil70778915	**Moesin**	1.37	**1.27**	NS	
54	gil6679567	**Polymerase I and transcript release factor**	1.43	**1.34**	NS	
55	gil31791059	**Activated leukocyte cell adhesion molecule**	1.60	**1.30**	NS	
56	gil76779293	**Keratin 8**	2.57	**1.21**	NS	
57	gil6671549	**Peroxiredoxin 6**	2.79	**1.32**	NS	
58	gil6680836	**Calreticulin**	4.22	**1.47**	NS	
59	gil29244550	**Transglutaminase 4 (prostate)**	8.08	**1.84**	NS	Suppressor
60	gil8567372	**Probasin**	8.76	**2.01**	NS	
61	gil2506545	**78-kDa glucose-regulated protein precursor (GRP 78)**	4.79	**1.53**	NS	
62	gil7643979	**170-kDa glucose regulated protein GRP170**	4.65	**1.51**	NS	
63	gil31542563	**DnaJ (Hsp40) homolog, subfamily C, member 3**	5.30	**1.49**	NS	
64	gil71774133	**Peptidylprolyl isomerase B (PPI)**	4.08	**1.32**	NS	
65	gil130232	**Protein disulfide isomerase b family, ERp57 subfamily**	3.82	**1.24**	NS	
66	gil129729	**Protein disulfide isomerase ERp59**	3.23	**1.28**	NS	
67	gil86198316	**Protein disulfide isomerase associated 4**	6.20	**1.49**	NS	
68	gil58037267	***Protein disulfide isomerase-associated 6***	5.97	***1.33***	***0.78***	
69	gil6753010	***Anterior gradient 2***	7.45	***1.50***	***0.71***	
70	gil42794267	***Experimental autoimmune prostatitis antigen 2***	15.01	***1.48***	***0.78***	Suppressor
71	gil31543942	***Vinculin***	1.27	***1.31***	***1.35***	
72	gil94387119	***PREDICTED: similar to heat shock protein 8***	1.49	***1.23***	***1.30***	
73	gil6752952	***Actin, γ 2, smooth muscle, enteric***	1.86	***1.28***	***1.78***	
74	gil94381948	***PREDICTED: similar to Fc fragment of IgG binding protein***	2.21	***1.55***	***1.28***	
75	gil6754482	***Keratin complex 1, acidic, gene 18***	3.71	***1.50***	***1.68***	

Abbreviation: NS, not significant.

*Proteins modulated by both MSeA and MSeC are shown in bold and italic font.

†Proteins modulated only by MSeA are shown in bold font.

‡Not significantly different from TRAMP group (ratio of 0.8-1.2 of the TRAMP group).

§Proteins modulated only by MSeC are shown in italic font.

were downregulated (Table 1B) in comparison with that of the wild-type mice. For example, proteins whose abundance was increased by over 2-fold in the TRAMP DLP compared with the wild-type counterpart included prosaposin (no. 12), Annexin A1 (no. 20), and 10 other proteins (no. 1, 3, 10, 11, and 16-22). The two most upregulated proteins were high mobility group box 2 (no. 16) and solute carrier family 12 protein (no. 17). Although the function of solute carrier family 12 protein in cancer is at present not clear, it is involved in spermatogenesis (24). In several cancer types, the human *high mobility group box2* (*HMGB2*) gene was significantly upregulated in cancerous tissues and was linked to cancer progression (25) and, therefore, may be an oncoprotein.

On the other hand, proteins downregulated in TRAMP by <2-fold included GSTM1 (Table 1B, no. 46; Figs. 2A and 3A), GSTM2 (Table 1B, no. 47), calponin-1 (Table 1B, no. 45; Fig. 3B), Annexin A6 (no. 48), lumican (no. 49), and transgelin (no. 50), and most proteins that were attenuated by MSeC only (no. 39-44) and several proteins that were attenuated by MSeA only (no. 53-55). Proteins with expression levels suppressed >2-fold in the TRAMP DLP compared with wild-type mouse DLP included mostly those modulated by MSeA (no. 56-70). Those suppressed by > 7-fold included transglutaminase 4 (TGM4, no. 59), probasin (no. 60), anterior gradient 2 protein (no. 69), and experimental autoimmune prostatitis antigen-2 (EAPA2, no. 70). Most of these proteins are related to prostate functional differentiation or AR signaling.

The identified proteins with altered expression in TRAMP belong to different functional family classes: prostate differentiation and AR signaling such as probasin [down in TRAMP, consistent with published data on protein as well as mRNA from other groups (refs. 26, 27)], calreticulin (no. 58, down in TRAMP), and seminal vesicle secretion-6 (no. 9, up in TRAMP); protein folding (misfolding) and endoplasmic reticulum (ER) stress [GRP78, peptidylprolyl isomerase B, PDI family, and GRP170 (no. 61-68); all down in TRAMP]; phase II detoxification/cytoprotective proteins [GSTM1 and GSTM2 (no. 46-47), down in TRAMP] and stromal cell–related proteins [calponin1 (no. 45), transgelin (no. 50), and caldesmon 1 (no. 40), all down in TRAMP] as well as proteins involved in other biological pathways (prosaposin and HMGB2). In summary, the iTRAQ approach identified both upregulated and suppressed proteins in the TRAMP DLP belonging to many functional categories. The implication of some of these proteins as potential oncogenes or suppressor genes will be further dealt with in Discussion.

Proteins modulated only by MSeA or MSeC revealed major biological differences

Among the proteins that were upregulated in TRAMP DLP (Table 1A; some of which might function as oncoproteins, see Discussion section), MSeA significantly suppressed the abundance of hemopexin (no. 8) and seminal vesicle secretion 6 (no. 9), and normalized that of seminal vesicle protein secretion 2, biliary glycoprotein, apolipoprotein A-1, brain abundant membrane attached signal protein 1, and cysteine-rich protein 1 (intestinal; no. 2-4, 6-7). MSeC treatment suppressed the abundance of biliary glycoprotein (no. 3), apolipoprotein A-1 (no. 4), proteasome (prosome, macropain)-28 subunit α, prosaposin, FUSE-binding protein 2, nucleolin, and tropomyosin 3, γ (no. 11-15, respectively). In contrast to MSeA for a complete reversion of the upregulation of seminal vesicle protein secretion 2 (no. 2), MSeC further enhanced its expression in the TRAMP mice.

Specific attenuation by each selenium form was even more apparent for suppressed proteins in the TRAMP DLP compared with the wild-type mouse counterpart (some of which could be tumor suppressors, see Discussion section; Table 1B). MSeA partially restored the expression of proteins related to prostate functional differentiation and AR signaling, e.g., probasin, calreticulin (28, 29), protein folding (misfolding), and ER stress responses, e.g., GRP78, GRP170, peptidylprolyl isomerase B, different PDI family proteins and DnaJ (Hsp40) homologue, subfamily C, member 3 (no. 58-68). MSeA treatment also partially restored some prostate-related proteins such as prostate TGM4 (no. 59), anterior gradient 2 (no. 69), and EAPA2 (70). As discussed later, TGM4 (30) and EAPA2 (28, 31) may function as tumor suppressor proteins. Additional proteins that specifically responded to MSeA included keratin 8 (no. 56) and peroxidoxin 6 (no. 57).

On the other hand, MSeC treatment did not affect the above proteins toward restoration of expression (Table 1B). In several cases, MSeC even further suppressed them, e.g., anterior gradient 2 (no. 69) and EAPA2 (no. 70). Instead, MSeC treatment restored the abundance of phase II detoxification and cytoprotective proteins including GSTM1, GSTM2 in full or in major extent (no. 46-47), and stromal cell–related proteins such as caldesmon 1 (no. 40), calponin1 (no. 45), and transgelin (no. 50). As a general trend, proteins specifically responding to MSeC attenuation seemed to be those with less extent of downregulation in the TRAMP model than those responding to MSeA. Our data implicate the restoration of those proteins, many of which are classic tumor suppressors such as GSTs, by MSeC, but not by MSeA, might be a specific mechanism for the chemopreventive effect of MSeC.

Very few TRAMP-regulated proteins were changed by both MSeA and MSeC in the same direction

Two proteins that were upregulated in the TRAMP mouse DLP compared with wild-type mouse DLP were reversed by both MSeA and MSeC: biliary glycoprotein (no. 3) and apolipoprotein A-1 (no. 4). Both Se forms exacerbated the overexpression of deleted in malignant brain tumors 1 isoform c (Table 1, no. 1). Similarly, very few of the downregulated proteins in TRAMP DLP were partially restored by both MSeA and MSeC, e.g., vinculin (Table 1, no. 71) and acidic keratin complex 1-gene 18 (no. 75).

Impact of MSeA or MSeC on proteins that were not significantly altered in TRAMP DLP

Although the abundance of several proteins was not different between TRAMP and wild-type mouse DLP, most of these proteins were again modulated by MSeA versus MSeC in distinct directions (Table 2). In particular, seminal vesicle protein 2, caltrin, malate dehydrogenase 2, and perhaps α-1-antitrypsin 1-6 precursor (Serine protease inhibitor 1-6) were significantly suppressed by MSeA and were not affected by MSeC (Table 2, no. 76-79). MSeA also suppressed serine protease inhibitor kazal-like protein (no. 81), whereas MSeC only had a modest reduction effect on it. MSeC, on the other hand, modestly induced aldolase A, myosin, light polypeptide kinase, and carbonic anhydrase 3 (Table 2, no. 83-85).

Discussion

In spite of extensive studies, the molecular mediators of TRAMP carcinogenesis remain to be fully elucidated. Literature review suggests some of the proteomic changes we observed here are consistent with the upregulation of classic onco-protein functions or suppression of classic tumor suppressors associated with TRAMP carcinogenesis, whereas the functional significance of many other proteins is at this moment not clearly defined. Among the 75 TRAMP-related proteins identified in this study, the same patterns of alterations have been reported for apolipoprotein A-1, ERp44, ERp57, ERp59, calreticulin, GRP78, PDIA4, PDIA6 (29), and probasin (27) through proteomic (two-dimensioanal electrophoresis) and transcriptomic approaches.

Probasin (Table 1B, no. 60) is a marker of differentiation and androgen action in the prostate. In the mouse, specific polyclonal antibodies against probasin showed primary localization to the apical membrane of differentiated secretory epithelium. Probasin expression in the prostate epithelial cells is mediated by AR transactivating the probasin promoter, whereas in the NE lineage cells in which AR is not expressed, its expression is most likely activated by Foxa family of transcriptional factors. Previous studies have shown that probasin mRNA and protein levels were absent in poorly differentiated TRAMP tumors by Johnson et al. (26). The mRNA level of probasin has also been reported by Haram et al. (27) to be dramatically decreased in TRAMP tumors at ~30 weeks. These studies did not distinguish NE carcinoma from other lesions. In a recent study on 11-week-old rats, mRNA levels of probasin and TGM4 (no. 59) and peroxidoxin 6 (no. 57) in the dorsal prostate were dramatically decreased after castration and were partially restored upon testosterone treatment (32). Our observed 8-fold reduction of probasin and TGM4 in the DLP of 18-week-old TRAMP mice compared with wild-type mice were consistent with the published work and support the overgrowth of precancerous prostate epithelial cells with less AR-mediated functional differentiation.

With immunohistochemistry staining and immunofluorescence assays, Birckbichler et al. (30) showed that expression and activity of TGM4 in prostate adenocarcinoma was significantly lower than normal prostate, benign prostatic hyperplasia, and inflammation tissues. The tumor suppressor PTEN induces TGM4 expression. Members of this family of proteins have been known

Table 2. Proteins that were not significantly changed in TRAMP DLP and their modulation by MSeA or MSeC

Entry no.	Access no.	Protein	iTRAQ abundance ratio		
			Wild: TRAMP	MSeA: TRAMP	MSeC: TRAMP
76	gil33468869	**Seminal vesicle protein 2***	NS†	**0.28**	NS
77	gil9931973	**Caltrin**	NS	**0.65**	NS
78	gil31982186	**Malate dehydrogenase 2, NAD (mitochondrial)**	NS	**0.77**	NS
79	gil68068019	**α-1-Antitrypsin 1-6 precursor (Serine protease inhibitor 1-6)**	NS	**0.78**	NS
80	gil21595014	***Ubiquinol-cytochrome c reductase binding protein‡***	NS	***0.73***	***0.69***
81	gil26388003	***Serine protease inhibitor kazal-like protein***	NS	***0.38***	***0.71***
82	gil13384620	*Heterogeneous nuclear ribonucleoprotein K§*	NS	NS	*0.77*
83	gil7548322	*Aldolase A*	NS	NS	*1.22*
84	gil55930915	*Myosin, light polypeptide kinase*	NS	NS	*1.24*
85	gil31982861	*Carbonic anhydrase 3*	NS	NS	*1.29*

*Proteins modulated by MSeA only are shown in bold font.
†Not significantly different from TRAMP group (ratio of 0.8-1.2 of the TRAMP group).
‡Proteins modulated by both MSeA and MSeC are shown in bold and italic font.
§Proteins modulated only by MSeC are shown in italic font.

to be involved in cross-linking proteins in apoptotic bodies and therefore may facilitate apoptosis and act as a tumor suppressor.

Murine experimental autoimmune prostatitis antigen EAPA2 (no. 70) was first identified by Setiady et al. (31) from anterior prostate lobes by immunoprecipitation with serum antibodies from mice with autoimmune prostatitis. Interestingly, EAPA1, another antigen identified at the same time with EAPA2, turned out to be TGM4 (31). 5α-Dihydrotestosterone treatment restored the expression of EAPA2 in the anterior prostate of neonatal orchiectomy B6AF1 mice. Fujimoto et al. (28) reported that EAPA2 was secreted by mouse dorsolateral and anterior prostate lobes. Further reverse transcription-PCR experiments indicated that the expression of EAPA2 was significantly reduced by castration but was partially reversed upon testosterone propionate treatment, which was consistent with the results by Setiady et al. (31). Although the biological function of EAPA2 has not been thoroughly reported yet, it could play an important role in maintaining the normal function of the prostate and may function as a tumor suppressor.

GSTM1 (no. 46) has been reported to be silenced in human prostate cancer due to hypermethylation in the promoter regions with a frequency as high as 58% (33). Several case-control studies have shown that GSTM1-null genotype, a genetic polymorphism with lower expression, was associated with increased prostate cancer risk (34, 35). A recent clinical proteomic study by Garbis et al. (36) also identified GSTM1 as a protein whose expression level was decreased in prostate cancer compared with benign prostatic hyperplasia tissues. These data suggest a role of loss of these phase II detoxification enzyme/cyto-protective proteins (tumor suppressors) in human prostate cancer etiology. Our findings are consistent with GSTM loss during TRAMP carcinogenesis.

Epithelial-stromal cell interactions play an important role in the etiology and progression of cancers, including prostate cancer (37, 38). A couple of stromal cell–related proteins were identified in this study. Calponin-1 (Table 1, no. 45) is a late-stage smooth muscle differentiation marker in prostate (37, 38). Its expression was lower in clinical benign prostatic hyperplasia compared with normal prostate transition zone tissues (39). Expression levels of calponin-1 and caldesmon 1 (Table 1, no. 40) are strong in normal prostates but are markedly reduced or even absent in the malignant tissues obtained from radical prostatectomy (40). Transgelin (Table 1, no. 50), another stromal marker, has also been reported to be a tumor suppressor involved in several biological pathways, such as tumor invasion and AR signaling (41).

On the other hand, prosaposin overexpression has been reported to stimulate AR and prostate-specific antigen expression in prostate cancer LNCaP cells (42). Its gene amplification and overexpression were observed in prostate cancer (43). Its upregulation in TRAMP DLP suggests a possible onco-protein property.

In terms of the effects of oral intake of the two chemopreventive forms of Se, MSeA versus MSeC, our data presented above suggest that biological differences in their molecular targets or mediators far outweighed similarities. Each affected a unique set of onco-proteins and tumor suppressors. As far as functional activities go, we and others have shown that MSeA suppresses AR level and signaling to prostate-specific antigen in LNCaP cell culture-model (44, 45). This might be consistent with the observed effect of MSeA on prostate differentiation and AR signaling proteins observed by proteomics here. In addition to the prostate differentiation and AR-regulated signaling proteins, many of the MSeA-modulated proteins are involved in protein folding (misfolding) and ER stress responses such as PDIs, PPIB, and GRP170. Some of them have been reported to be expressed or functionally related to prostate cancer (28, 29, 32, 46). Expression of PDI was significantly reduced upon castration in male mouse DLP lobe and partially reversed upon testosterone treatment (28), and the same trends for GRP78 were observed in rats recently (32). GRP170, the largest ER resident chaperone, enhanced the therapeutic effect of tumor suppressor mda-7/interleukin 24 against TRAMP C2 allografts in a gene therapy trial (47). Based on a rapid suppression of vascular endothelial growth factor, matrix metalloproteinase-2, and prostate-specific antigen by MSeA in different cell model systems and their common characteristic of being secretory proteins with disulfide bonds, we have speculated that MSeA could induce unfolded protein response in the ER to lead to their rapid protein degradation (11). Ip and coworkers (48) have shown that PC-3 cells exposed to MSeA in cell culture display ER stress responses including GRP78 and CHOP (Oncovin)/gadd153. Taken together, these findings suggest that MSeA may specifically restore ER stress rescue and chaperone-like proteins in the prostate epithelial cells to contribute to the inhibition of carcinogenesis.

Chemically and metabolically, MSeA is monomethylated seleninic acid whereas MSeC is a selenoamino acid (11), and both have been postulated to be precursors of methylselenol (Fig. 1C). Consistent with the distinct proteome responses affected by MSeA versus MSeC in the carcinogenic prostate, we have also observed very different protein profiles affected by MSeA versus MSeC in the normal prostate tissue from mice treated with these Se forms (49). We confirmed that MSeC, but not MSeA, upregulated GSTM1, and MSeA, but not MSeC, upregulated EAPA2 in the normal prostate. These data support the biological differences and specificity of each form of methylated Se compounds in normal and carcinogenic prostates.

In summary, although MSeA and MSeC were both considered as methylselenol precursors, the documented differences in protein profiles affected by each Se form suggest different targets, and mechanisms of action outweigh those shared through their common methylselenol pool. *In vivo* efficacy difference of MSeA versus MSeC against PC-3 xenograft as we reported previously (9) lend more support to this conclusion. The data suggest that

MSeA and MSeC should be developed as separate Se agents rather than as equal precursors of methylselenol, as the current dogma suggests.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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