行政院國家科學委員會專題研究計畫 成果報告

Transglutaminase 於培養細胞對高溫及氧化壓力反應所扮演之角色

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Transglutaminase於培養細胞對高溫及氧化壓力反應所扮演之角色

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一、中文摘要

轉穀胺醯氨酶摧化之反應基本上是以酵素Cys 的thiol group 去攻打Gln 上的carbonyl group，NH3離開，形成Thioester bond。而此Thioester bond 大多是接受Lys 的ε-amino group 攻擊形成Isopeptide bond 而酵素離開。此Isopeptide bond 亦可接受另一個一級氨、水分子或者醇類的攻擊。因此此酵素作用的反應為Transadmidation 造成蛋白質之聚合、Amine incorporation 造成polyamines共價結合上蛋白質、Esterification 造成Gln 轉換成酸酯、Deamidation 造成Gln 轉換成Glu、及Isopeptide cleavage 造成聚合蛋白質的分開。其主要功能在於凝血、瘍口癒口、調控細胞自戕死亡、神導傳導物質釋放、訊息傳導及細胞間質形成等。轉穀胺醯氨酶之功能必需透過受質之鑑定以及Transamidation 造成之影響才得以瞭解。

本實驗室以新穎方法來純化及鑑定轉穀胺醯氨酶之受質，小鼠(>10 周齡)之肝臟及睪丸其轉穀胺醯氨酶酵素活性，並純化出受質，通過質譜分析鑑定受質的身分，最後以免疫轉漬法以確認受質之真正身分。在超過一白個鑑定受質中，大概屬下列性質蛋白；細胞骨架及其調節蛋白、Chaperones 及Co-chaperones、內質網蛋白、細胞Detoxification用蛋白、蛋白質轉譯調節蛋白等，大多是與細胞壓力反應有關之蛋白。更重要的是其中多個受質屬新的發現。由於轉穀胺醯氨酶本身是氧化壓力所活化的酵素，而且多數受質屬壓力反應有關之蛋白，因此我們認為轉穀胺醯氨酶及其受質参與細胞壓力反應，其扮演之角色則是本計畫探討的主題。

關鍵詞：轉穀胺醯氨酶、質譜分析、受質

Abstract

Transglutaminases (TG) are Ca^{2+}-dependent enzymes which catalyze a post-translational modification of proteins. The enzyme reaction leads to the formation of an isopeptide bond either within or between polypeptide chains. The γ-glutamyl-ε-lysine crosslinks are formed between the γ-carboxamide group of peptide-bound glutamine residues and the ε-amino group of peptide-bound lysine residues. Polyamines can replace lysine residue in the transamidation reaction in vitro and in vivo. Transglutaminases are abundant enzymes which are involved in a number of different physiologic processes, e.g., plasma transglutaminase factor XIII stabilizes the fibrin clot during hemostasis, and keratinocyte and epidermal transglutaminase contribute to the formation of the cornified envelope in skin. Several physiological roles for tissue transglutaminases have been demonstrated, such as wound healing, fibrosis, apoptosis, and matrix formation.

In order to understand the physiological functions of tissue transglutaminase (tTG or TG2), one needs to identify the acyl donor and the acyl acceptor substrates in the transamidation reaction. To this end, we have identified 29 potential transglutaminase substrates from mouse liver extract by substrate purification and tandem Mass Spectrometry analysis. Interestingly, chaperones and co-chaperones are the most abundant tTG substrates found. Other transglutaminase substrates include intermediate filament protein, β-actin, proteasome proteins, peroxiredoxin, 14-3-3 proteins, valosin-containing protein, nucleolin, glyoxalase 1. Many of the tTG substrates are cellular proteins with functions related to cellular response to stress. More importantly, some of the tTG substrates have not been reported. In light of the fact that oxidative stress or UV irradiation elevates *in situ* tTG activity, we seek to study the role of tTG in HepG2 cells in response to heat shock and oxidative stress. More specifically, we will determine whether tTG is up-regulated or activated in HepG2 cells in response to heat shock and oxidative stress and further to determine the consequences of tTG activation. In addition, we will examine whether the tTG substrates that we have identified in mouse liver extract are indeed modified in vivo in HepG2 cells. In the end, we wish to know the roles of tTG and heat shock proteins and their interplay in HepG2 cells in response to heat shock and oxidative stress.
Keywords: transglutaminase、substrates、proteomics、Mass spectrometry

二、緣由與目的

轉穀胺醯氨酶家族有三組成員：Papain-like transglutaminases、protein disulfide isomerase-like transglutaminases及bacterial toxin transglutaminases。Papain-like transglutaminases屬於Cysteine protease

superfamily的一員，其它次家族成員有papain、calpain、foot and mouth virus protease、deubiquitylating enzymes、及N-acetyltransferases (Laszlo and Graham, 2003)。這些酵素具有與摧化反應相關的結構如Cys-His-Asn或Cys-His-Glu，稱catalytic triad。

人類全基因含九個transglutaminase基因，其中有一個是erythrocyte band4.2已不具摧化能力，其詳細特性如表一所列。已知的蛋白質均無醣化修飾，也缺乏雙硫鍵，摧化機制需要鈣離子(mM)的存在。Type 2 transglutaminase(TG2)是第一個被發現的成員，幾乎表現在所有的器官，也表現在細胞的不同部位如cytosol (80%)、plasma membrane (含extracellular matrix，10-15%)、nuclear membrane (5%)。雖然一級結構缺乏Signal peptide，TG2卻可以分泌到細胞表面及細胞間質上。最奇特的是它又是個GTP-binding protein，結合GTP會抑制transglutaminase酵素活性，但是它又具GTPase活性，GTP終究水解為GDP。不過若TG2先結合了鈣離子，鈣離子可阻斷GTP的結合。因此TG2與高分子量G protein (Ghα)是同一個蛋白質，可媒介αadrenergic receptor活化所造成之phospholipase C的活化(Nakaoka et al., 1994)。

轉穀胺醯氨酶之受質鑑定有一種較傳統的方法，是把純化蛋白加入酵素看蛋白質是否聚合，或再加入5-(biotinamido)pentylamine為探針去標示蛋白質，不過這種作法之生理意義較受質疑，實驗靠內生性酵素作反應較有意義。轉穀胺醯氨酶之受質鑑定有二個對象；一稱Acyl donor指提供Gln參與反應的蛋白質，另一稱Acyl acceptor指提供Lys參與反應的蛋白質，此二對象一般是二個不同之蛋白質亦可以是同一個蛋白質，形成Intramolecular isopeptide bond，或是Heterodimer。所以在受質鑑定就有二群對象；以5-(biotinamido)pentylamine為探針(或^{3}[H]-putrescine)，經摧化反應後可標示到Acyl donor，此標示之蛋白質可轉漬至PVDF後以Streptavidin-peroxidase呈色(或以X光片)顯示，或經Streptavidin-agarose親和力管柱純化。至於Acyl acceptor之鑑定文獻上則有不同的biotinylated peptide的設計，其中序列中含Gln氨基酸；如LGLGQGKVLG (Gorman and Folk, 1984)、TVQQEL (Ruoppolo et al., 2003)、GQQQLG (Hu and Messersmith, 2003)、QQIV (Lorand et al., 1992)等。Pastor等(1999)曾對此設計作過詳細探討，結論如下：1. Gln不可在N-或C-terminus、2. Gln不可位在二個鹼性胺基酸中間或Pro中間。在文獻上報導的Acyl donor較多，可能是因為5-(biotinamido)pentylamine及^{3}[H]-putrescine較易取得，同時也不必作設計。針對這問題，作者已設計合成了一個新的探計Biotin-RSGQQQLGSS，反應極佳而且水溶解度非常好，因此本計畫將以此胜肽及5-(biotinamido)pentylamine為探針，尋找及鑑定轉穀胺醯氨酶之受質。

在本計畫中，我們利用小鼠的睪丸為材料，以蛋白質體學的方法去純化以及鑑定小鼠睪丸內TG的受質，希望在一個像testis這樣持續進行細胞生長與分化的組織裡，了解TG所扮演的角色為何。經由純化及MS/MS鑑定過後，我們發現了數十種蛋白質在小鼠睪丸內可能為TG的受質，在經由重組TG受質蛋白的in vitro transamidation反應以及TG substrates pull-down的immunoblotting assay，我們也證實了這些蛋白在in vitro的確為TG的受質。未來我們希望從中選取了幾個可能為TG受質的蛋白，觀察其TG進行transamidation過後，會對這些蛋白的酵素活性、protein-protein interaction或是protein translocation會有怎樣的影響，進而能更加了解TG在testis內的角色與功能。

三、材料與方法

蛋白質膠體電泳

電泳配方及條件參考Schägger & Jagow (1987)所著Tricine-SDS方法，Running gel之濃度為7.5%，stacking gel濃度為4%，Cathode buffer組成是0.1 M Tris、0.1M Tricine、0.1% SDS，pH 8.25。Anode buffer組成是0.2 M Tris, pH 8.9。Gel buffer組成是1 M Tris、0.1% SDS，pH 8.45。電泳條件是五十伏特二十分鐘及一百伏特七十分鐘。之後膠體以水洗六十分鐘後以Colloidal Coomassie blue G-250 染色六十分鐘，再以水洗淨二十分鐘。銀染色則採取Amersham-Pharmacia Plus One Silver Stain Kit，並依原廠商提供步驟進行染色，但是退染改為5% 醋酸．

近免疫轉漬法 (Near-immunoblotting)

將欲分析之材料進行膠體電泳分析，利用Diffusion blotting (Chen and Chang, 2001)將蛋白質轉印至PVDF濾紙上。以Phosphate buffered saline (PBS)洗滌十分鐘，加入Blocking solution (5% skim milk in PBS, 0.05% Tween 20)室溫反應一小時，再以PBS洗滌三次。接著加入Rabbit IgG-streptavidin complex (1 ug/ul於PBS，含0.05% Tween 20、3 mg/ml BSA)於室溫反應一小時，再以PBS-T洗滌三次，然後加入Peroxidae-conjugated anti-rabbit IgG抗體(溶於PBS含0.05% Tween、3 mg/l BSA)反應於室溫一小時，再以PBS洗滌三次。最後以DAB (0.6 mg/ml)、0.01% 氯化鎳、1 ul/ml 雙氧水呈色。

轉穀胺醯氨酶摧化反應

我們取成鼠Testis以10重量體積之20 mM Tris, pH 8, and 2% Triton X-100溶液研磨後，以20,000 xg離心三十分鐘分成分上清液及不溶物二部分。不溶物再以20 mM Tris, pH8, and 0.15M NaCl (TBS)清洗一次。轉穀胺醯氨酶摧化反應含0.2 ug/ul 5-(biotinamido)pentylamine (bPA) 或 biotin-RSGQQQLGSS (bPQ) 以及5 mM $CaCl_2$, and 0.1 mM DTT及適量之組織材料。反應於室溫進行三十分鐘，有時加入20 mM of dansylcadaverine當作抑制劑。上清液以加入等體積之2x SDS sample buffer中止反應。而不溶物靠加入

Urea到8M濃度，混合十分鐘後離心取得可溶蛋白。

轉穀胺醯氨酶受質純化

因為要作純化所以上清液中止反應改成加入Urea到8M、DTT到50 mM，於室溫一小時後加入Iodoacetamide到100 mM，再置於室溫一小時。之後材料以Amersham Biosciences出產之2-D clean-up kit作蛋白質沈澱，後溶於8M urea/TBS中，與等體積之Streptavidin-argarose beads (以8 M urea/TBS平衡)結合，後以五倍體積8 M urea/1.0 M NaCl/TBS, 0.1% SDS/TBS, 及20mM Tris buffer清洗Streptavidin-agarose beads各一次，最後以二倍體積4% SDS並加熱將受質沖洗出來。

西方墨點法(Western blotting)

將欲分析之材料進行膠體電泳分析，利用Semi-dry blotter (Hoeffer Semi-Phor)將蛋白質轉印至PVDF濾紙上。以Phosphate buffered saline (PBS)洗滌十分鐘，加入Blocking solution (5% skim milk in PBS)室溫反應一小時，再以PBS洗滌三次。接著加入抗血清(一比一千稀釋於PBS，含1 mM EDTA、3 mg/ml BSA)於攝氏四度反應十六小時，再以PBS洗滌三次，然後加入Peroxidase-conjugated anti-guinea pig IgG抗體(溶於PBS含1 mM EDTA、3 mg/l BSA)反應於室溫二小時，再以PBS洗滌三次。最後以DAB (0.6 mg/ml)、0.01% 氯化鎳、1 ul/ml 雙氧水呈色。

質譜分析 (Mass spectrophotometry analysis and protein in-gel digestion)

將純化得到之蛋白質作SDS電泳，以Coomassie blue染色，待腿色後，以刀片將色帶切下，放入微量管中，加入100 µl之DTT/25 mM碳酸氫氨。於37ºC反應一小時，加入100 µl Acetonitrile，振盪數分鐘，離心除去上清液。加入100 µl 65 mM Iodoacetamide/25 mM 碳酸氫氨，於室溫反應一小時，加入100 µl Acetonitrile，振盪數分鐘，離心除去上清液。加入200 µl 50% Acetonitrile/25 mM 碳酸氨，振盪數分鐘，離心除去上清液。加入200 µl 100% Acetonitrile，振盪數分鐘，離心除去上清液。加入10-15 µl trypsin 溶液(0.1-0.15 µg配於25 mM碳酸氨)，於37ºC反應16小時，加入10 µl 100% Acetinitrile，振盪數分鐘，離心收集上清液。殘餘膠體加入20 µl 100% Acetonitrile/0.1% TFA萃取，振盪數分鐘，離心收集上清液。殘餘膠體加入20 µl 50% Acetonitrile/0.1% TFA萃取，振盪數分鐘，離心收集上清液。殘餘膠體加入20 µl 100% Acetonitrile/0.1% TFA萃取，振盪數分鐘，離心收集上清液。把各次收集之上清液混合，並以SpeedVac抽至殘留體積約10 µl，送中央研究院基因體/蛋白質體中心，以LC-Mass-Mass作蛋白質定序。

四、結果與討論

我們不將mouse cytosol直接進行in vitro transamidation的反應，再利用ammonium sulphate做沉澱，以8 M urea/20 mM Tris-HCl 回溶沉澱後的蛋白，並利用streptavidin argerose進行純化步驟。最後純化到的binding proteins，經電泳及Coomassie blue染色解析後，將SDS-PAGE上的lane（包含bPQ和bPA）對分為二十等分，操作in-gel digestion，將tryptic peptides由膠片中萃取出，接著進行MS/MS的分析（圖一），鑑定出的蛋白身分詳列於表一。經由蛋白質質譜鑑定，最後鎖定12個我們有興趣研究的TG substrates，進行重組蛋白的製備。所選用的表現載體為pET21b，使用*E. coli* BL21作為宿主，表現我們所挑選出蛋白的部分片段。預測這幾個蛋白的分子量理論值：LDH testis isoform為36 kDa、NSFL1 cofactorp47為41 kDa、nudC為38 kDa、Peroxiredoxin-1為22 kDa、Retinal dehydrogenase為54 kDa、TOM34為34 kDa、Glutathione S-transferase Mu 5為27 kDa、HSP70為70 kDa、APG-1為94 kDa、Poly(A)-binding protein 1為71 kDa、Heterogeneous nuclear ribonucleoprotein K為51 kDa、hnRNP A2/B1為36 kDa。 在這裡我們盡量去表現出全長的蛋白，但其中HSP70與APG-1僅表現出1/3與1/2的長度，這些表現蛋白在經過induction以及純化之後，接著進行in vitro transamidation以及biotin overlay assay。由結果顯示（圖二），在有外加TG的條件下，這些重組蛋白會有bPA incorporation的情形發生，有些甚至會有高分子量的complex形成，懷疑是由於TG的cross-linking作用而將其重組蛋白本身作了cross-linking所導致。將純化到之binding proteins，進行SDS-PAGE及轉印後，以各分子的抗體血清操作西方轉印法（圖三）。由圖三結果我們可以發現，albumin, nudc, hsp70, tubulin, apg1及hsp90在沒有加入任何的biotin probe sample裡，看不到訊號的產生，而在加入bPQ或是bPA的sampl裡，可以看到有蛋白存在的訊號。

在此次的TG substrate純化與鑑定中，我們一共使用了兩種不同的probes（bPA：5-biotin-amidopentylamine、bPQ：biotin-SGQQQLGSS ），其中bPA作為一個K donor，提供TG反應時所需的amino group，bPQ作為一個Q donor，提供TG反應時所需的carboxamide group。所以本次實驗經由MS/MS的分析結果，有些蛋白是TG利用bPA所鑑定到的受質，有些則是TG利用bPQ所鑑定到的受質，代表這些蛋白有些是利用本身的帶有的carboxamide group與bPA反應，有些則是利用本身的amino group與bPQ進行反應。而有些蛋白同時被鑑定為bPA和bPQ都可反應的分子時，代表這類的分子本身應該同時具有Q donor和K donor，而這樣的分子本身就容易被TG作用，產生oligomer。像是Poly A-binding protein 1、Heat shock protein 70.2、Retinal dehydrogenase、Glutathione S-transferase 1、Peroxiredoxin

等。

TG substrates bPA in testis	Molecular size	Function	Mascot score	Fragments found	Accession #
Serum albumin	67 kD	Serum protein	407	14	P07724
Alpha-2-macroglobulin	220 kD	Protease inhibitor	218	4	Q61838
Pyruvate carboxylase	130 kD	Endogenous biotin-conjugated protein	197	6	Z14044
Clathrin heavy chain	90 kD	Endocytosis	116	5	Q68FD5
(HLA-B-associated transcript 3)	83 kD	Contains ubiquitin-like domain	87	1	Q6ZQ38
Poly(A)-binding protein1	71 kD	Pre-mRNA splicing	592	17	P29341
Heterogeneous nuclear ribonucleoprotein U-like protein 1	96 kD	mRNA processing, transcription repressor	124	2	Q8VDM6
Glial fibrillary acidic protein,	50 kD	Glial intermediate filament	61	5	P03995
Tubulin alpha-6 chain	50 kD	Microtubule component	111	3	P68373
Heat shock 70 kDa protein 4	94kD	HSP 70 family chaperone	151	5	Q61316
Hexokinase-1	108 kD	Glycolysis enzyme	56	2	P17710
Protein KIAA1967 homolog	102 kD	Unknown	67	2	Q8VDP4
Heat shock protein HSP 90-alpha	86 kD	Molecular chaperone	769	17	P07901
Heat shock protein HSP 90-beta	86 kD	Molecular chaperone	408	11	P11499
Piwi-like protein 1	98 kD	RNA-binding protein	183	6	Q9JMB7
Microtubule-associated protein 4	117 kD	Promotes microtubule assembly	133	3	P27546
Polypyrimidine tract-binding protein-associated-splicing factor)	75 kD	Pre-mRNA splicing	73	2	Q8VIJ6
Sperm equatorial segment protein 1	45 kD	Acrosome protein	67	3	Q9D5A0
Heat shock-related 70 kDa protein 2	70 kD	Molecular chaperone	435	10	P17156
Dipeptidase 3	54 kD	GPI-anchored dipeptidase	83	2	Q9DA79

TG substrates bPA in testis	Molecular size	Function	Mascot score	Fragments found	Accession #
RNA-binding protein EWS	68 kD	Possible transcription repressor	79	3	Q61545
L-amino acid oxidase	70 kD	Lysosome enzyme	70	2	O09046
Matricin	70 kD	TCP-1 gamma chaperone	112	3	P80318
RNA-binding protein FUS	53 kD	Nuclear riboprotein	92	3	P56959
Elongation factor Tu	50 kD	Protein translation	128	5	P10126
Alpha-2-antiplasmin	55 kD	Serine protease inhibitor	84	4	Q61247
Propionyl-CoA carboxylase alpha chain	80 kD	Endogenous biotin-conjugated protein	70	3	Q91ZA3
Retinal dehydrogenase 1	54 kD	Aldehyde dehydrogenase	153	5	P24549
UV excision repair protein RAD23 homolog B	43 kD	DNA repair	115	3	P54728
Heterogeneous nuclear ribonucleoprotein K	51 kD	Pre-mRNA splicing	241	7	P61979
Y-box-binding protein 2	38 kD	Pre-mRNA splicing	166	4	Q9Z2C8
T-complex protein 1 subunit alpha A	60 kD	Molecular chaperone	73	3	P11984
Nucleoporin-like protein RIP	58 kD	Acrosome biogenesis	68	3	Q8K2K6
Alpha-tubulin 3/7	50 kD	Microtubule component	415	9	P05214
GPI-anchored protein p137	73 kD	Unknown	116	1	Q60865
Drebrin-like protein	49 kD	F-actin binding	60	2	Q62418
NSFL1 cofactor p47	41 kD	Golgi fragmentation and reassembly	457	8	Q9CZ44
ATP-dependent RNA helicase eIF4A-1	46 kD	Protein translation	106	2	P60843
Septin-2 (Protein NEDD5)	41 kD	Cytokinesis	72	2	P42208
AU-rich element RNA-binding protein 1	38 kD	mRNA turnover	56	3	Q60668
Beta-actin	42 kDa	Microfilament component	435	8	P60710
hnRNP X	38 kDa	RNA binding	202	4	Q61990

40S ribosomal protein SA	33 kDa	Ribosomal protein	113	3	P14206
LDH testis isoform	36 kDa	Glucose metabolism	936	31	P00342
APC-binding protein EB1	30 kDa	Microtubule and spindle formaiton	121	3	Q61166
14-3-3 protein epsilon	29 kDa	Phosphoprotein binding	202	4	P62259
TG substrates bPA in testis	Molecular size	Function	Mascot score	Fragments found	Accession #
14-3-3 protein theta	28 kDa	Phosphoprotein binding	139	3	P68254
14-3-3 protein zeta/delta	28 kDa	Phosphoprotein binding	135	4	P63101
Tubulin beta-2C	50 kDa	Microtubule component	134	4	P68372
LDH-A	36 kDa	Glucose metabolism	128	9	P06151
DEAD box RNA helicase DEAD1	69 kDa	Pre-mRNA splicing	114	2	Q61656
Peroxiredoxin-4	31 kDa	Redox regulation	146	4	O08807
Glutathione S-transferase Mu 5	27 kDa	Testis GST	220	8	P48774
hnRNP-E1	37 kDa	RNA binding	117	2	P60335
snRNP-B	24 kDa	Pre-mRNA splicing	83	2	P27048
hnRNP F	45 kDa	Pre-mRNA splicing	80	4	Q9Z2X1
Glutathione S-transferase A4	26 kDa	Cell detoxification	158	6	P24472
Peroxiredoxin-2	21 kDa	Redox regulation	112	3	Q61171
hnRNP H	49 kDa	Pre-mRNA splicing	112	3	O35737

TG substrates bPQ in testis	Molecular size	Function	Mascot score	Fragments found	Accession #
Serum albumin	67 kD	Serum protein	418	9	P07724
Pyruvate carboxylase	129 kD	Endogenous biotin-conjugated protein	140	6	Q05920
Alpha-tubulin 3/7	50 kD	Microtubule component	291	7	P05214
Clathrin heavy chain	191 kD	Endocytosis	98	2	Q68FD5
MAP 4	118 kD	Microtubule assembly	65	2	P27546
Elongation factor Tu	50 kD	Protein translation	371	12	P10126
GRP94; HSP 90 beta	92 kD	Molecular chaperone	336	9	P08113
HSP 90 alpha	85 kD	Molecular chaperone	708	15	P07901
Hsp 75	80 kD	Mitochondria chaperone	96	2	Q9CQN1
Beta-actin	42 kD	Microfilament component	415	10	P60710
eIF-4B	69 kD	Protein translation	117	3	Q8BGD9

Nucleolin	77 kD	Nucleolar protein	100, 72	2, 2	P09405
Spermatid-specific thioredoxin-1	58 kD	Spermatogenesis	89	2	Q6P902
Transferrin	77 kD	Fe (III) binding	63	2	Q921I1
GFAP	50 kD	Glial intermediate filament	63	2	P03995
HSP 70.2	70 kD	Molecular chaperone	564	14	P17156
Heat shock cognate 71 kDa protein	71 kD	Molecular chaperone	364	7	P63017
Cortactin	61 kD	Src substrate	227	6	Q60598
Propionyl-CoA carboxylase alpha chain	80 kD	Endogenous biotin-conjugated protein	166	4	Q91ZA3
DEAD box RNA helicase DEAD1	69 kD	Pre-mRNA splicing	118	3	Q61656
Heat shock 70 kDa protein 4L	94 kD	Molecular chaperone induced by osmotic stress	86	2	P48722
Kininogen-1	73 kD	Cys protease inhibitor	128	4	O08677
Y-box-binding protein 2	38 kD	mRNA processing	62, 95	2, 1	Q9Z2C8
Retinal dehydrogenase 1	54 kD	Aldehyde dehydrogenase	330	7	P24549
Tubulin beta-2C	49 kD	Microtubule component	245	7	P68372
ALDH class 2	56 kD	Aldehyde dehydrogenase	183	6	P47738
PAI mRNA-binding protein 1	45 kD	mRNA stability	94	1	Q9CY58
Unc-33-like phosphoprotein)	62 kD	Synaptic vesicle protein	66	2	Q62188
hnRNP F	46 kD	Pre-mRNA splicing	165	6	Q9Z2X1

TG substrates bPQ in testis	Molecula r size	Function	Mascot score	Fragments found	Accession #
Proacrosin-binding protein sp32	61 kD	Acrosome packaging	137	3	Q3V140
Argininosuccinate synthase	46 kD	Urea cycle enzyme	110	3	P16460
hnRNP H	49 kD	Pre-mRNA splicing	80	2	O35737
Testis-specific gene A2	34 kD	Male miosis	196	4	Q8VIG3
hnRNP A3	40 kD	Pre-mRNA splicing	109	1	Q8BG05
Septin-2 (NEDD5)	42 kD	Cytokinesis	96	2	P42208
Leucine-rich repeat-containing protein 46	36 kD	Unknown	74	2	Q9DAP0

nudC	38 kD	Mitotic spindle formation	191	6	O35685
GAPDH	36 kD	Glycolysis enzyme	397	8	P16858
Suppressor of G2 allele of SKP1 homolog	38 kD	SCF complex component	127	3	Q9CX34
hnRNP A2/B1	36 kD	Pre-mRNA splicing	202	4	O88569
hnRNP C1 / C2	34 kD	Pre-mRNA splicing	89	2	Q9Z204
TOM34	34 kD	Mitochondria protein import	494	14	Q9CYG7
Protein phosphatase 1C catalytic subunit	37 kD	Signal transduction	84	2	P63087
LDH testis subunit	36 kD	Glycolysis	837	29	P00342
Swiprosin-1	27 kD	Calcium binding	146	4	Q9D8Y0
Nuclear protein Hcc-1	23 kD	Nucleic acid binding	92	2	Q9D1J3
TCP-1-theta	59 kD	Molecular chaperone	88	2	P42932
eIF4E	25 kD	Protein translation	61	2	P63073
Heat shock 70 kDa protein 4L	94 kD	Molecular chaperone	86	2	P48722
14-3-3 protein epsilon	29 kD	Phosphoprotein binding	285	5	P62259
14-3-3 protein zeta/delta	28 kD	Phosphoprotein binding	182	4	P63101
eIF-4H	27 kD	Protein translation	92	1	Q9WUK2
Peroxiredoxin-1	22 kD	Redox regulation	405	13	P35700
HSP 27	23 kD	Molecular chaperone	124	2	P14602
AKAP 82	93 kD	Sperm motility	83	2	Q60662
14-3-3 protein beta/alpha	28 kD	Phosphoprotein binding	73	2	Q9CQV8
14-3-3 protein sigma	28 kD	Phosphoprotein binding	73	2	O70456
TG substrates bPQ in testis	Molecula r size	Function	Mascot score	Fragments found	Accession #
Peroxiredoxin-2	21 kD	Redox regulation	235	5	Q61171
Glutathione S-transferase Mu 1	26 kD	Cell detoxification	189	7	P10649
Peroxiredoxin-4	31 kD	Redox regulation	118	3	O08807
GST class-mu 5	26 kD	Cell detoxification	393	9	P48774
GST A4-4	25 kD	Cell detoxification	152	6	P24472
GST class-mu 6	25 kD	Cell detoxificatin	108	6	O35660
Poly(A)-binding protein 1	70 kD	Pre-mRMA splicing	107	3	P29341
Stathmin	18 kD	Microtubule destabilization	72	2	P54227

hnRNP F	50 kD	Pre-mRNA processing	128	4	Q9Z2X1

五、參考文獻

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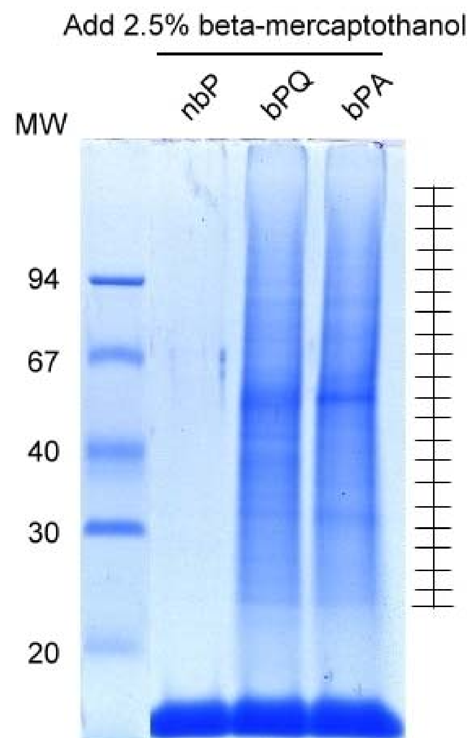

Figure 1. **Purification of bPQ- and bPA- incorporated TG substrates from mouse testis cytosol.**

Mouse testis crude extract was processed by in vitro transamidation. The biotin-labeled TG substrates were purified by streptavidin beads and separated by SDS-PAGE. Polyacrylamide gel of each lane was divided equally into 20 parts and subjected to in-gel digestion and LC-MS/MS analysis.

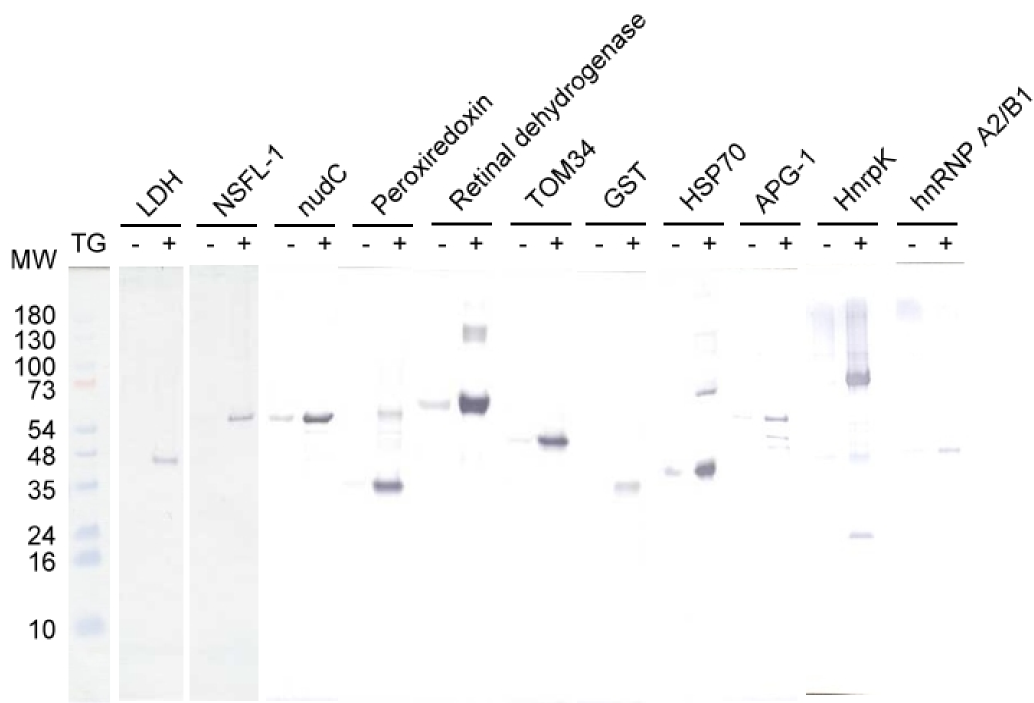

Figure 2. In vitro transamidation of recombinant TG substrates.
Recombinant TG substrates were processed by in vitro transamidation with biotinamido-pentylamine and biotin overlay assay. (LDH: Lactate dehydrogenase; NSFL1: NSFL1 cofactor p47; nudC: nucleo distribution protein C; translocase of the outer membrane of mitochondria； GST: Glutathione S-transferase 1; HSP70: Heat shock protein 70.2; Poly A: Poly A binding protein 1; HnrpK: Heterogeneous nuclear ribonucleoprotein K; hnRNP A2/B1: Heterogeneous ribonucleoprotein particles)

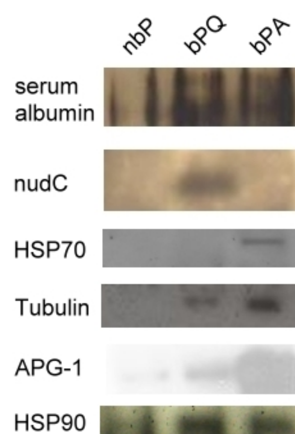

Figure 3. Western blotting of biotin-labeled TG protein substrates.