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Identification of three pertussis toxin substrates (41, 40 and 39 kDa proteins) in mammalian brain

Comparison of predicted amino acid sequences from G-protein α-subunit genes and cDNAs with partial amino acid sequences from purified proteins

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We have determined the partial amino acid sequences of the 40 kDa protein, one of the three pertussis toxin substrates in porcine brain. Purified 40 kDa protein from porcine brain was completely digested with TPCK-trypsin. Digested peptides were separated by reverse-phase HPLC and subjected to analysis by gas-phase protein sequencing. Several sequences of porcine brain 40 kDa protein completely matched with those which were deduced from the nucleotide sequences of the human $G_i2\alpha$ gene and rat $G_i2\alpha$ cDNA. On the other hand, the previously determined sequences of the rat brain 41 and 39 kDa proteins were in complete agreement with the predicted amino acid sequences of rat $G_i1\alpha$ and $G_o\alpha$ cDNAs, respectively.

Pertussis toxin substrate; Amino acid sequence; G-protein; Signal transduction; 40 kDa protein

1. INTRODUCTION

In many signal-transducing systems, G-proteins function as transducers [1]. G-proteins are a family of guanine-nucleotide-binding proteins which are structurally homologous and widely distributed in eukaryotic cells. G-proteins are heterotrimers composed of α-, β- and γ-subunits. The α-subunit which binds GTP is unique to each G-protein, while the β- and γ-subunits are similar, if not identical. Recently, molecular cloning of G-protein α-subunit genes and cDNAs has been achieved [2–14]. Besides two transducin α-subunits ($G_t1\alpha$, $G_t2\alpha$), the existence of at least four genes for pertussis toxin substrates has been demonstrated. The multiplicity of pertussis toxin substrates at the protein level had already been reported [15,16]. In mammalian brain, three substrates differ in the apparent molecular mass on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of their α-subunits, i.e. the 41, 40 and 39 kDa proteins. More recently, each substrate has been purified separately from porcine brain and the biological and immunochemical properties studied [16]. Here, we describe the identification of three mammalian brain substrates by determination of the partial amino acid sequence and comparison with the predicted amino acid sequences from G-protein α-subunit genes and cDNAs.

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2. MATERIALS AND METHODS

2.1. *Purification of 40 kDa protein from rat and porcine brain*

The procedures employed to purify three pertussis toxin substrates from porcine brain membranes are described by Katada et al. [16]. Briefly, a mixture of pertussis toxin substrates after DEAE-Toyopearl 650 (S) chromatography were applied to a Mono Q HR5/5 column and eluted with NaCl gradients as in [16]. Each of three peaks was pooled, diluted, and further purified by rechromatography on the Mono Q column. The pertussis toxin substrates from rat brain membranes were also purified using the above procedure. Resolution of pertussis toxin substrates to the GTPγS-bound α-subunit and the βγ-subunits was performed by high-performance gel filtration using a TSK 3000 SW column as in [17].

2.2. *Trypsin digestion of porcine brain 40 kDa protein*

A sample containing approx. 0.5 nmol purified 40 kDa protein was concentrated from 1 ml to 250 μl in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 200 mM NaCl, 0.7% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), 20 μM $AlCl_3$, 6 mM $MgCl_2$, 10 mM NaF using centricon 10 (Amicon). Concentrated samples were heated at 65°C for 15 min and digested at 37°C with tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin. TPCK-treated trypsin was added at a 40 kDa protein/trypsin ratio of 40% (w/w). After incubation for 12 h, the same amount of TPCK-treated trypsin was added, and the incubation continued for an additional 12 h.

2.3. *Purification of tryptic peptides*

Following tryptic digestion of porcine brain 40 kDa protein, insoluble material was removed by centrifugation. The supernatant was diluted with 0.1% trifluoroacetic acid and 5% acetonitrile, and applied to a column of PepRPC HR5/5 equilibrated with the same solvent. The material was eluted with a linear gradient of 5–35% acetonitrile at a flow rate of 0.7 ml/min using a Pharmacia FPLC system. The eluate absorbance at 210 nm was recorded with a Jasco Uvidec-100-V ultraviolet monitor. Four fractions (I–IV) corresponding to absorbance peaks were collected (fig.2).

2.4. *Sequence analysis of peptides*

Four fractions were subjected to amino acid sequence analysis with a gas-phase sequencer (model 470A, Applied Biosystems). Phenylthiohydantoin (PTH) amino acids were analysed by high-performance liquid chromatography (HPLC) using a Hitachi HPLC system.

3. RESULTS

To identify the three major pertussis toxin substrates of mammalian brain, we purified the substrates from porcine and rat brain membranes. Fig.1 shows the SDS-PAGE analysis of the three purified pertussis toxin substrates. The molecular masses of the three α-subunits were found to be 41, 40 and 39 kDa. They were ADP-ribosylated by pertussis toxin (not shown). For sequence analysis, the porcine brain αβγ complex containing 40 kDa protein was resolved into the 40 kDa protein and

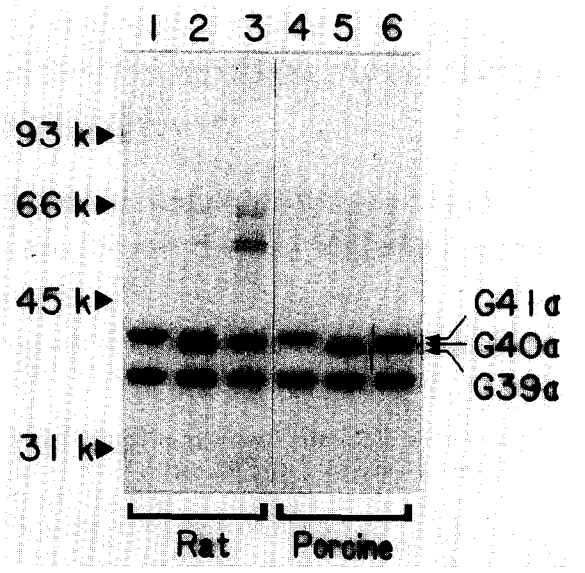

Fig.1. SDS-polyacrylamide gel electrophoresis of pertussis toxin substrates purified from porcine and rat brain membranes. Proteins were purified from rat (lanes 1–3) and porcine (lanes 4–6) brain membranes, and subjected to SDS-polyacrylamide gel (10%) electrophoresis and staining with Coomassie blue.

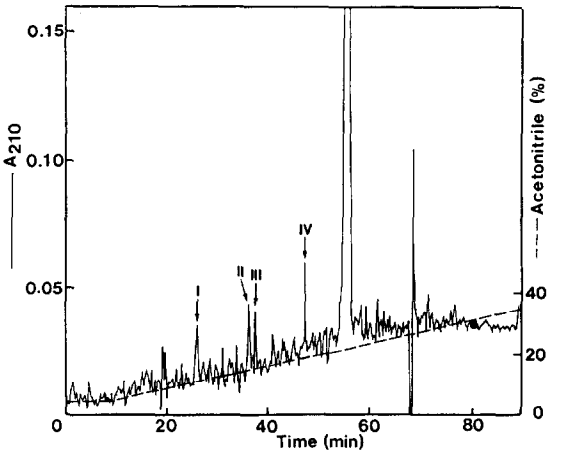

Fig.2. Reverse-phase HPLC analysis of tryptic peptides from porcine brain 40 kDa protein. Tryptic peptides of the 40 kDa protein were applied to a Pep HR5/5 column and eluted as described in section 2. Four peaks (I–IV) were subjected to amino acid sequence analysis.

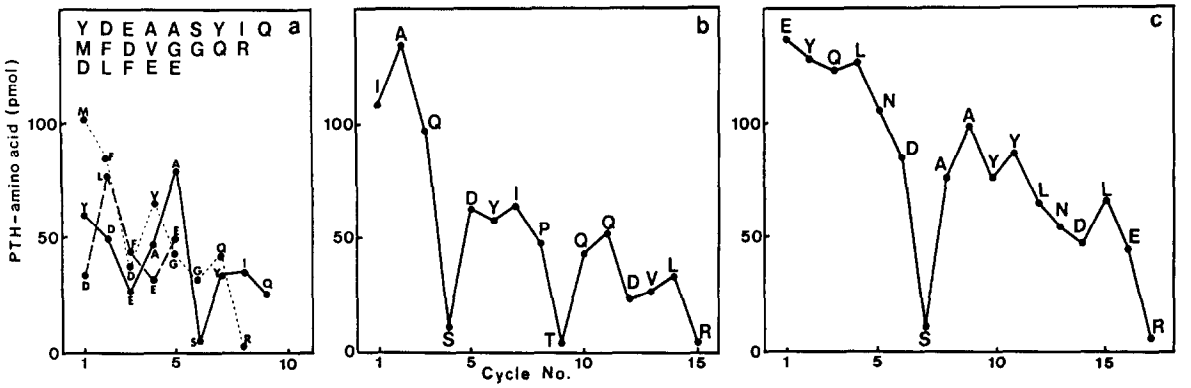

Fig.3. Partial amino acid sequence analysis of the porcine brain 40 kDa protein. Fractions I (a), II (b), III and IV (c) were subjected to sequence analysis with a gas-phase sequencer. The yield of PTH-amino acids at each cycle of Edman degradation is shown. The one-letter amino acid notation is used. Fraction I was a mixture of three peptides (I-1–I-3).

porcine	**G40α**	**(I-1)**	**K/R**	**Y**	**D**	**E**	**A**	**A**	**S**	**Y**	**I**	**Q**			
human	Gi2α	(296-305)	K	Y	D	E	A	A	S	Y	I	Q			
rat	Gi2α	(296-305)	K	Y	D	E	A	A	S	Y	I	Q			
mouse	Gi2α	(296-305)	K	Y	D	E	A	A	S	Y	I	Q			
human	Gi3α	(295-304)	T	Y	E	E	A	A	A	Y	I	Q			
rat	Gi3α	(295-304)	T	Y	E	E	A	A	A	Y	I	Q			
human	Gi1α	(295-304)	T	Y	E	E	A	A	A	Y	I	Q			
bovine	Gi1α	(295-304)	T	Y	E	E	A	A	A	Y	I	Q			
rat	Gi1α	(295-304)	T	Y	E	E	A	A	A	Y	I	Q			
rat	Goα	(296-305)	T	Y	E	D	A	A	A	Y	I	Q			
bovine	Goα	(296-305)	T	Y	E	D	A	A	A	Y	I	Q			

porcine	**G40α**	**(II)**	**I**	**A**	**Q**	**S**	**D**	**Y**	**I**	**P**	**T**	**Q**	**Q**	**D**	**V**	**L**	**R**
human	Gi2α	(163-177)	I	A	Q	S	D	Y	I	P	T	Q	Q	D	V	L	R
rat	Gi2α	(163-177)	I	A	Q	S	D	Y	I	P	T	Q	Q	D	V	L	R
mouse	Gi2α	(163-177)	I	A	Q	S	D	Y	I	P	T	Q	Q	D	V	L	R
human	Gi3α	(162-176)	I	S	Q	S	N	Y	I	P	T	Q	Q	D	V	L	R
rat	Gi3α	(162-176)	I	S	Q	T	N	Y	I	P	T	Q	Q	D	V	L	R
human	Gi1α	(162-176)	I	A	Q	P	N	Y	I	P	T	Q	Q	D	V	L	R
bovine	Gi1α	(162-176)	I	A	Q	P	N	Y	I	P	T	Q	Q	D	V	L	R
rat	Gi1α	(162-176)	I	A	Q	P	N	Y	I	P	T	Q	Q	D	V	L	R
rat	G41α	(I)	I	A	Q	P	N	Y	I	P	T	Q	Q	X	V	L	
rat	Goα	(163-177)	I	G	A	A	D	Y	Q	P	T	E	Q	D	I	L	R
bovine	Goα	(163-177)	I	G	A	A	D	Y	Q	P	T	E	Q	D	I	L	R

porcine	**G40α**	**(IV)**	**E**	**Y**	**Q**	**L**	**N**	**D**	**S**	**A**	**A**	**Y**	**Y**	**L**	**N**	**D**	**L**	**E**	**R**
human	Gi2α	(146-162)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	E	R
rat	Gi2α	(146-162)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	E	R
mouse	Gi2α	(146-162)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	E	R
human	Gi3α	(145-161)	E	Y	Q	L	N	D	S	A	S	Y	Y	L	N	D	L	D	R
rat	Gi3α	(145-161)	E	Y	Q	L	N	D	S	A	S	Y	Y	L	N	D	L	D	R
human	Gi1α	(145-161)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	D	R
bovine	Gi1α	(145-161)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	D	R
rat	Gi1α	(145-161)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	D	R
rat	G41α	(II)	E	Y	Q	L	N	D	S	A	A	Y	Y	L	N	D	L	X	R
bovine	Goα	(146-162)	E	Y	Q	L	N	D	S	A	K	Y	Y	L	D	S	L	D	R
rat	Goα	(146-162)	E	Y	Q	L	N	D	S	A	K	Y	Y	L	D	S	L	D	R
rat	G39α	(I & II)	E	Y	Q	L	N	D	S	A		Y	Y	L	D	S	L	D	

Fig.4. Amino acid sequence homology among the G-protein α-subunits. The sequences of the G-protein α-subunits have been taken from the literature: human $G_i2\alpha$ [13], rat $G_i2\alpha$ [3], mouse $G_i2\alpha$ [4], human and rat $G_i3\alpha$s [13], human $G_i1\alpha$ [9,13], bovine $G_i1\alpha$ [2], rat $G_i1\alpha$ [11], rat $G_o\alpha$ [3], bovine $G_o\alpha$ [7]. The partial amino acid sequences of rat brain 41 kDa (G41α) and 39 kDa (G39α) proteins are described in [3]. In the predicted sequences, amino acid residues are numbered beginning with the initiating methionine of the respective polypeptides. The two sequnces (G39α-I and -II) of rat brain 39 kDa protein are EYQLNDSA and YYLDSLD, respectively. In the porcine G40α (I-1) sequence, the NH_2-terminal amino acid residue is assumed to be K or R (K/R). X, unidentified residue. Differences, as compared with the residues of the porcine brain 40 kDa protein, are boxed.

$\beta\gamma$-subunits as described in section 2. Resolved 40 kDa protein was digested with TPCK-trypsin, and the cleavage products separated by reverse-phase HPLC (fig.2). Four fractions (I–IV) corresponding to the absorbance peaks were collected and subjected to amino acid sequence analysis. Fig.3 shows the sequences derived from three peak fractions (I, II, IV). Fractions II–IV contained a single peptide, while fraction I was a mixture of three peptides. The sequences determined were as follows: I-1, YDEAASYIQ; I-2, MFDVGGQR; I-3, DLFEE; II, IAQSDYIPTQQDVLR; III, TTGIVETH; IV, EYQLNDSAAYYLNDLER. Assignment of the sequences of the three peptides in fraction I was based on the predicted amino acid sequences from the known G-protein α-subunit genes and cDNAs [2–13].

We compared the partial amino acid sequences derived from porcine brain 40 kDa protein with those predicted from G-protein α-subunit genes and cDNAs (fig.4). The sequences of I-2, I-3 and III matched completely those which were identical in all different members of the $G_i\alpha$ subfamily (not shown) [13]. In contrast, the sequences of I-1, II and IV which were derived from porcine brain 40 kDa protein (G40α) were identical with those of human [13], rat [3] and mouse [4] $G_i2\alpha$, but not with those of $G_i1\alpha$, $G_i3\alpha$ and $G_o\alpha$.

4. DISCUSSION

In this report, we determined the partial amino acid sequences of the six tryptic peptides containing 62 amino acid residues of porcine brain 40 kDa protein. The results indicated that the porcine 40 kDa protein was different from $G_i1\alpha$, $G_i3\alpha$, and $G_o\alpha$, but identical with $G_i2\alpha$ (fig.4). In [3], we had determined the partial amino acid sequences of rat brain 41 kDa (G41α) and 39 kDa (G39α) proteins. The partial amino acid sequences of the six tryptic peptides of 60 amino acid residues from rat brain G39α were in complete agreement with those predicted for rat $G_o\alpha$ cDNA. One of the amino acid sequences, MEDTEPFSAELLSAMM, was unique to $G_o\alpha$. On the other hand, in one of the seven tryptic peptides of rat brain G41α, rat G41α (I), the amino acid sequence Pro-Asn was different from Ser-Asp (166–167) predicted from the nucleotide sequence of rat $G_i2\alpha$ cDNA [3]. However, this particular amino acid sequence was present in rat [11], bovine [2] and human [9] $G_i1\alpha$ sequences as shown in fig.4. From genetic analysis

Table 1

Summary of molecular cloning of G_i and G_o α-subunit genes and cDNAs

DNA library	Classification and nomenclature				Reference
Genomic library					
Human	$G_i1\alpha$ (?)	$G_i2\alpha$ (355)	$G_i3\alpha$ (354)		[13]
cDNA library					
Human brain	α_i-1 (?)				[9]
Human T-cells		α_i2 (355)	α_i3 (354)		[12]
Human monocytes (U-937)		$G_i\alpha$ (355)			[6]
Human granulocytes (HL-60)			Gxα (354)		[8]
Human liver			α_i-3 (?)		[10]
Bovine cerebral cortex	$G_i\alpha$ (354)				[2]
Bovine pituitary gland	α_i (?)	(αh ?[a])			[5]
Bovine retina				$G_o\alpha$ (354)	[7]
Rat glioma cells (C6)		$G_i2\alpha$ (355)	$G_i3\alpha$ (354)	$G_o\alpha$ (354[b])	[3,13]
Rat olfactory epithelium	$G\alpha_i1$ (354)	$G\alpha_i2$ (355)	$G\alpha_i3$ (354)	$G\alpha_o$ (354)	[11]
Mouse macrophages (PU-5)		α_i (355)			[4]
Sizes of mammalian brain					
G-protein α-subunits	41 kDa	40 kDa	?	39 kDa	

[a] A cDNA clone, which apparently differs from all of the cDNA and genomic clones described above, was also reported

[b] Full-length sequence unpublished

Numbers in parentheses indicate the length of the deduced amino acid sequences of each clone

[2–14] strong conservation of the amino acid sequence in each group of the G-protein α-subunit family was found. There were homologies of greater than 98% among each $G_i\alpha$ subfamily. The amino acid sequence of $G_s\alpha$ is strongly conserved between human and rat, only one out of 394 amino acids being different [14]. This is supported by immunological analysis using specific anti-Gα antibodies. Indeed, the antibodies raised against rat brain 41 and 39 kDa proteins reacted specifically with porcine brain 41 and 39 kDa proteins, respectively, but did not cross-react with porcine brain 40 kDa protein [16]. These results suggest that the three major pertussis toxin substrates in mammalian brain, the 41, 40 and 39 kDa proteins, may correspond to $G_i1\alpha$, $G_i2\alpha$ and $G_o\alpha$, respectively. However, the protein encoded by the $G_i3\alpha$ gene has not yet been identified. Our preliminary work suggests that there is at least one additional pertussis toxin substrate in mammalian brain. We shall attempt to purify and characterize this protein.

The 40 kDa pertussis toxin substrate is widely distributed in cells and tissues from different species. In mammalian brain, the order of relative abundance of three pertussis toxin substrates in $G39\alpha > G41\alpha > G40\alpha$. On the other hand, in other tissues and cells such as neutrophils [18] and C6 glioma cells [19], the molecular mass of the major pertussis toxin substrate amounted to 40 kDa. Anti-brain 41 kDa and anti-brain 39 kDa antibodies did not cross-react with their substrates. We previously reported that the $G_i2\alpha$ gene seemed to be a housekeeping gene [13]. What is the physiological role of $G_i2\alpha$ in signal transduction? More recently, Murayama and Ui [20] reported the involvement of a pertussis toxin substrate in proliferation of 3T3 cells. It is of interest that significant homology was found between the 5′-flanking region of the human $G_i2\alpha$ gene and the human c-Ha-*ras* gene [13]. The $G_i2\alpha$ gene product, $G40\alpha$, might function in a new slow signal-transduction system leading to cell proliferation.

During preparation of this manuscript, sequence analysis of cDNA and genomic clones for the $G_i\alpha$ subfamily and $G_o\alpha$ was carried out independently in several laboratories as shown in table 1 [2–13]. The nomenclature of the three $G_i\alpha$ subspecies, $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$, tentatively designated by Gilman [1] and Suki et al. [10], is used here. Once the function of each $G_i\alpha$ subspecies and $G_o\alpha$ has been ascertained, the nomenclature should be settled.

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