Glycosylation and transmembrane topography of bovine chromaffin granule p65

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The bovine homologue of p65, a calmodulin-binding protein located in the membranes of synaptic vesicles and endocrine secretory granules, has been studied by the use of monoclonal antibodies directed against this antigen and against dopamine β -mono-oxygenase. The protein (apparent molecular mass 67 kDa; pI = 5.5–6.2) is partially degraded by treatment with neuraminidase or endoglycosidase F. Trypsin treatment of intact adrenal chromaffin granules or of granule membranes releases a soluble 39 kDa fragment of p65 which corresponds to the whole of its cytoplasmic domain. This domain contains both the epitope for the monoclonal antibody cgm67 and the calmodulin-binding site. The 20 amino acids at the *N*-terminus of this fragment are identical to part of the rat p65 sequence.

INTRODUCTION

Although the process of regulated secretion is reasonably well characterized in pharmacological terms, its molecular details have yet to be elucidated. One approach is to use monoclonal antibodies, raised against secretory granule membranes, in the biochemical characterization of proteins on the granule surface. By this means several granule antigens have been recognized, although their functions have yet to be fully defined; these include p38 (synaptophysin; Wiedenmann & Franke, 1985), SV2 (Buckley & Kelly, 1985) and p65 (Matthew *et al.*, 1981).

This last protein was first identified as a component of rat brain synaptic vesicles, and monoclonal antibodies against it were found to precipitate chromaffin granules from bovine adrenal medulla (Lowe *et al.*, 1988). Subsequently p65 was shown to be identical with a 65 kDa calmodulin-binding protein that had already been identified in the membranes of chromaffin granules (Fournier & Trifaró, 1988; Fournier *et al.*, 1989*b*). The amino acid sequence of rat p65, deduced by sequencing a cDNA clone (Perin *et al.*, 1990), suggested that the protein had a single membrane-spanning sequence; its *N*-terminal domain contained a single putative *N*-glycosylation site and was predicted to lie within the lumen of the granule, whereas the cytoplasmic *C*-terminal domain contained two related sequences with sequence similarity to a regulatory domain of protein kinase C.

We now report some studies on the structure and topography of p65, using a monoclonal antibody (cgm67) raised against bovine chromaffin granules. Our results provide direct biochemical evidence in support of the structural model of Perin *et al.* (1990).

MATERIALS AND METHODS

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland, and purified by the method of Bordier (1981). All other reagents were of AnalaR grade. Monoclonal antibody asv48 was kindly given by Dr. John Bixby (University of Miami, Miami, FL, U.S.A.).

Preparation of chromaffin granule membranes

Chromaffin granules were isolated from fresh bovine adrenal glands (obtained from the local slaughterhouse) by the method

of Phillips (1974), and purified granule membranes were prepared from them according to Apps & Schatz (1979). All procedures were carried out at 4 °C in media buffered with 10 mM-Hepes/NaOH, pH 7.2, and containing 2 mM-EGTA, 1 mM-benzamidine and 0.2 mM-phenylmethanesulphonyl fluoride (PMSF). The membranes were finally resuspended in 10 mM-Hepes/NaOH (pH 7.2)/5 mM-dithiothreitol (DTT) to a protein concentration of 10 mg/ml.

Triton X-114 fractionation of chromaffin granule membranes

Chromaffin granule membranes were resuspended to 4 mg of protein/ml in Tris-buffered saline (20 mM-Tris/150 mM-NaCl), pH 7.4, with 2% (w/v, final) Triton X-114, and fractionation was carried out according to Pryde & Phillips (1986). Most experiments were carried out using the 'aqueous' phase, which contains residual Triton X-114. Removal of this detergent precipitated the chromaffin granule membrane glycoproteins, producing the 'glycoprotein-rich' fraction.

pH 11 treatment of membranes

Chromaffin granule membranes (approx. 1 mg/ml) were suspended in 0.1 M-Na₂CO₃, pH 11, containing 10 mM-EDTA. After 30 min at 0 °C the membranes were separated from solubilized proteins by centrifugation.

Preparation of antibodies

cgm67 and dbh1 monoclonal antibodies were prepared essentially as described by Bastin *et al.* (1982). Mice were immunized with the glycoprotein-rich Triton X-114 fraction of chromaffin granule membranes. Hybridomas were screened against the immunogen.

Calmodulin-affinity chromatography of chromaffin granule membrane glycoproteins

The aqueous fraction from Triton X-114 fractionation of chromaffin granule membranes was incubated for 20 min with calmodulin-agarose gel (Sigma) in 10 mM-Hepes/NaOH (pH 7.2)/0.1 mM-CaCl₂. After extensive washing in the same buffer, bound proteins were eluted by replacing CaCl₂ by 5 mM-EGTA. Controls were performed with the addition of 2 mM-EGTA during the binding incubation.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; Tos-Lys-CH₂Cl, *N*- α -tosyl-2-lysine chloromethyl ketone ('TLCK'); DTT, dithiothreitol.

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Enzymic deglycosylation of gp67

N-Deglycosylation was carried out using endoglycosidase F (EC 3.2.1.96; Boehringer) and desialylation was carried out with neuraminidase (sialidase, EC 3.2.1.18; Sigma, type VI). For deglycosylation, 100 μ g of the aqueous fraction from Triton X-114 fractionation of chromaffin granule membranes was lyophilized and resuspended with the following reagents to give a final protein concentration of 1.6 mg/ml: 50 mM-Mes/NaOH, pH 6.5, 0.5% deoxycholate, 1% Triton X-100, 5 mM-DTT, 15 mM-EDTA, pH 6.5, and 33 units of endoglycosidase F/ml. The digestion was carried out at 30 °C for 6 h in the presence of 5 mM-benzamidine, 200 μ M-PMSF, 5 μ g of leupeptin/ml, 5 μ g of pepstatin/ml and 50 μ g of Tos-Lys-CH₂Cl (*N*- α -*p*-tosyl-2-lysine chloromethyl ketone; TLCK)/ml. For digestion with neuraminidase, 100 μ g of the Triton X-114 aqueous fraction was lyophilized and resuspended in 50 mM-Mes/NaOH, pH 6.0, to a final concentration of 1.6 mg/ml in the presence of 0.2% Triton X-100, 5 mM-DTT and the above proteinase inhibitors. Neuraminidase was added to a final concentration of 1 unit/ml and the reaction mixture was incubated at 30 °C for 6 h. The samples were lyophilized and dissolved in SDS sample buffer with 5 mM-DTT for analysis by gel electrophoresis and immunoblotting.

Proteinase digestion of chromaffin granule membranes

Chromaffin granule membranes were resuspended to 1 mg/ml and incubated with various concentrations of trypsin for 60 min at 20 °C in the presence of 2 mM-DTT. The digestions were stopped by the addition of 50 μ g of Tos-Lys-CH₂Cl and 200 μ M-PMSF. The soluble peptides were separated from the membranes by centrifugation (412000 g_{av} , 15 min, 2 °C). The resulting pellet was washed twice by resuspension and centrifugation in 10 mM-Hepes/NaOH, pH 7.2, containing the same inhibitors. The supernatant fraction was recentrifuged as above to remove any residual membrane fragments. The membrane fractions were resuspended and then denatured by the addition of 0.25 vol. of hot (100 °C) pH 4.6 SDS sample buffer [0.2 M-potassium bipthalate, pH 4.6, 0.2 mM-EDTA, 20% (w/v) SDS, 40% (v/v) glycerol, 0.015% (w/v) Bromophenol Blue] in the presence of 5 mM-DTT. The soluble fractions were lyophilized and taken up in one-tenth of the original volume of 4-fold diluted SDS sample buffer (pH 4.6, as above). The samples were then analysed by electrophoresis and immunoblotting.

Electrophoresis and immunoblotting

One- and two-dimensional gel electrophoresis was performed as described by O'Farrell (1975) and by Gavine *et al.* (1984), with the exception that samples were lyophilized before use. Electrophoretic transfer to nitrocellulose was done according to Towbin *et al.* (1979). Incubation with primary antibody was carried out overnight, with bound antibody being detected either with ¹²⁵I-labelled second antibody or with biotinylated second antibody followed by ¹²⁵I-streptavidin.

Purification and partial amino acid sequencing of the cytosolic domain of p65

Chromaffin granule membranes (10 mg of protein in 10 ml of 10 mM-Hepes/NaOH, pH 7.2, 5 mM-DTT) were incubated with 10 μ g of trypsin for 60 min at 20 °C, and the reaction was stopped by the addition of 50 μ g of Tos-Lys-CH₂Cl/ml and 200 μ M-PMSF. The membranes were pelleted and the supernatant was removed and made 1.25 M in ammonium sulphate and 50 mM in sodium phosphate, pH 7.0. This sample was applied to a hydrophobic interaction column (Phenyl-5PW; TSK) equilibrated in the same medium, washed with this medium and eluted with a 30 ml linear gradient of 1.25–0 M-ammonium

sulphate in 50 mM-phosphate buffer, pH 7.0. Fractions were assayed for the cgm67 epitope by immunodotting, and active fractions containing an immunoreactive 39 kDa peptide were pooled, dialysed against distilled water and concentrated by rotary evaporation. Samples from six such runs were combined. The dialysed sample was made 10% in acetonitrile and 0.1% in trifluoroacetic acid, applied to a C₁₈ reverse-phase h.p.l.c. column and eluted with a linear gradient of 10–70% acetonitrile in water containing 0.1% trifluoroacetic acid. The peak containing the cgm67-immunoreactive 39 kDa peptide was checked for purity by gel electrophoresis and Coomassie Blue staining, and was sequenced on an Applied Biosystems type 477 microsequencer by automated Edman degradation.

RESULTS AND DISCUSSION

The cgm67 epitope is on p65

The non-ionic detergent Triton X-114 may be used to fractionate membrane proteins according to their hydrophobicities (Pryde & Phillips, 1986). After fractionation of bovine chromaffin granule membranes by this method, the glycoprotein-rich fraction was used as the immunogen in the preparation of a class IgM mouse monoclonal antibody, which was named cgm67. This antibody recognizes an epitope on a bovine chromaffin

Fig. 1. Detection of cgm67-reactive epitopes in chromaffin granule membranes

(a) Immunoblot of a two-dimensional electrophoretogram of chromaffin granule membranes using the monoclonal antibody cgm67. (b) Immunoblot showing the distribution of cgm67 immunoreactivity on fractionation of chromaffin granule membranes with Triton X-114 (tracks 1–6) or washing at pH 11 (tracks 7 and 8). Track 1, chromaffin granule membranes; track 2, phospholipid-rich fraction; track 3, detergent-rich fraction; track 4, aqueous fraction; track 5, glycoprotein-rich fraction; track 6, supernatant remaining after removal of glycoproteins from aqueous fraction; tracks 7 and 8, pellet and supernatant obtained on washing chromaffin granule membranes at pH 11. In each track 15 μ g of protein was loaded.

Fig. 2. Two-dimensional immunoblots

(a) and (b) show the position of protein p65 relative to that of dopamine β -mono-oxygenase (D β H, 75 kDa), and were developed with monoclonal antibodies cgm67 (a) and asv48 (b), together with dbh1, a monoclonal antibody directed against dopamine β -mono-oxygenase.

Fig. 3. Enzymic degradation of the oligosaccharide chains of p65

The aqueous fraction from Triton X-114 treatment of chromaffin granule membranes was treated with neuraminidase (track 2) or endoglycosidase F (track 3); the product was analysed by immune blotting with cgm67. Tracks 1 and 4 are controls, incubated without enzyme.

granule membrane protein of apparent molecular mass 67 kDa and pI 5.5–6.2 (Fig. 1a). The antigen was shown to be located in the granule fraction by subcellular fractionation, and its distribution paralleled that of dopamine β -mono-oxygenase (results not shown).

When chromaffin granule membranes were fractionated with Triton X-114, immunoreactivity with cgm67 appeared predominantly in the aqueous phase (Fig. 1b, track 4). Removal of residual Triton X-114 from this fraction concentrated the immunoreactivity in the insoluble glycoprotein-rich fraction (Fig. 1b, track 5), leaving the supernatant, which contained secretory proteins, devoid of immunoreactivity (Fig. 1b, track 6). The immunoreactive protein also remained membrane-bound on treatment with 100 mM- Na_2CO_3 , consistent with it being an integral membrane protein (Fig. 1b, track 7).

The apparent molecular mass of the cgm67-reactive protein is similar to that of p65, a 65 kDa protein of rat synaptic vesicle membranes (Matthew *et al.*, 1981) also shown to be one of the calmodulin-binding proteins of bovine chromaffin granule membranes (Fournier & Trifaró, 1988). To determine whether the protein recognized by cgm67 was indeed p65, we obtained a calmodulin-binding fraction from solubilized chromaffin granule membranes by calmodulin-agarose affinity chromatography (see the Materials and methods section), separated the proteins by two-dimensional electrophoresis and blotted them both with cgm67 and with asv48, the monoclonal antibody originally used

in characterizing p65 (Matthew *et al.*, 1981). A trace of dopamine β -mono-oxygenase was added to the material loaded on to the gel, and was used as an internal pI and molecular mass marker by detection with dbh1, a class IgG anti-(dopamine β -mono-oxygenase) monoclonal antibody. The positions of the asv48- and cgm67-immunoreactive spots were coincident, although that due to cgm67 was much stronger than that due to asv48 (Figs. 2a and 2b). As p65 has been shown to bind both asv48 and calmodulin and to be present in bovine chromaffin granule membranes (Fournier & Trifaró, 1988), we conclude that cgm67 binds to an epitope on this protein, although we found an apparent molecular mass of 67 kDa rather than the 65 kDa reported in the rat (Matthew *et al.*, 1981). Both of these values are much larger than the peptide molecular mass calculated from the rat sequence, which is 47576 Da.

Bovine chromaffin granule p65 is N-glycosylated

Incubation of the aqueous fraction of chromaffin granule membranes with endoglycosidase F or neuraminidase produced partial degradation of p65, as shown by SDS/PAGE. Neuraminidase, which removes terminal sialic acid residues from oligosaccharides, decreased the molecular mass of p65 by about 1 kDa (Fig. 3, tracks 1 and 2), whereas endoglycosidase F, which completely removes N-linked high-mannose and complex oligosaccharides, produced a greater decrease, of about 5 kDa (Fig. 3, tracks 3 and 4). We also found that p65 did not bind the lectins concanavalin A or wheatgerm agglutinin, but that it did bind peanut agglutinin after neuraminidase treatment (results not shown). Together, these results indicate that bovine p65 contains one or more N-linked oligosaccharide chains, terminating in sialic acid and galactose. The decrease in molecular mass on deglycosylation is not large enough to account for the discrepancy between the calculated molecular mass and that determined by SDS/PAGE (see above).

Isolation and partial amino acid sequencing of the cytosolic domain of bovine p65

Treatment of intact chromaffin granules or of their broken membranes with low concentrations of trypsin released soluble cgm67-reactive cleavage products, the major one being a fragment of about 39 kDa. As the trypsin concentration was increased from 0.1 to 20 $\mu\text{g}/\text{ml}$, more of the 39 kDa fragment was released (Figs. 4c and 4d, tracks 4–10), but at higher concentrations ($> 20 \mu\text{g}/\text{ml}$) this began to be degraded to a 27 kDa fragment (tracks 9–11). As the detection of the fragments depends upon their immunoreactivities, p65-derived polypeptides that do not contain the cgm67 epitope are not seen in these blots.

On trypsin treatment of broken membranes the amount of residual intact p65 decreased as the trypsin concentration was increased from 0.01 to 2.5 $\mu\text{g}/\text{ml}$ (Fig. 4b, tracks 1–6), and a new membrane-bound form of molecular mass 45 kDa appeared, with a smaller amount of a 28 kDa form being seen at trypsin concentrations around 200 $\mu\text{g}/\text{ml}$ (Fig. 4b, tracks 5–11). This 45 kDa species was not seen when intact granules were incubated with trypsin (Fig. 4a), nor was any other membrane-bound cgm67-immunoreactive product.

These results are consistent with the release of a large (39 kDa) domain of p65 into the soluble fraction from both broken membranes and intact granules and with the release of a smaller, luminal domain (22 kDa; non-immunoreactive) from broken membranes only. The cgm67 epitope is present on the larger domain, and this must be cytosolic, as it is released from intact chromaffin granules by trypsin, and no membrane-bound degradation product can then be detected (Fig. 4a). According to the structure prediction by Perin *et al.* (1990), this cytosolic domain is C-terminal to the membrane-spanning sequence (see Fig. 6).

Fig. 4. Trypsin degradation of p65

Intact chromaffin granules or chromaffin granule membranes were incubated with a range of trypsin concentrations ($\mu\text{g/ml}$: track 1, 0.01; track 2, 0.03; track 3, 0.09; track 4, 0.27; track 5, 0.81; track 6, 2.5; track 7, 7.4; track 8, 22; track 9, 67; track 10, 200; track 11, 600) and the insoluble and soluble fractions were examined separately by immune blotting with cgm67. Tracks marked + are controls without trypsin. (a) and (b) show insoluble fractions from intact granules and granules membrane respectively; (c) and (d) show soluble fractions from intact granules and granule membranes respectively.

Fig. 6. Diagram of the structure of bovine p65

T22, T28, T40, T50 and T55 are trypsin cleavage sites; boxes A and B indicate regions which in rat p65 contain sequences similar to protein kinase C. For the amino acid sequence, see Table 1.

Fig. 5. Binding of p65 and its cytoplasmic domain to calmodulin

The aqueous fraction from Triton X-114 treatment of chromaffin granule membranes (tracks 1 and 6) was incubated with immobilized calmodulin in the presence of $100\ \mu\text{M-Ca}^{2+}$ (total) and eluted with 5 mM-EGTA. Track 2, eluate; track 3, eluate when 2 mM-EGTA was included during the incubation. Tracks 4 and 5 show a similar experiment in which the cytoplasmic domain of p65, liberated by treatment of chromaffin granule membranes with trypsin (see Fig. 4d), was incubated with immobilized calmodulin. Proteins were visualized by immunoblotting with cgm67.

The 39 kDa tryptic fragment showed Ca^{2+} -dependent binding to immobilized calmodulin, from which it was eluted by EGTA (Fig. 5, track 4). An endogenous 39 kDa degradation product which was present in the aqueous phase from Triton X-114 fractionation (Fig. 5, track 1) behaved similarly (Fig. 5, track 2). A small amount of another (approx. 50 kDa) endogenous degradation product that was detectable in this fraction failed to bind to calmodulin (Fig. 5, tracks 1 and 2).

The 39 kDa tryptic fragment of p65 bound to a phenyl-5PW hydrophobic interaction chromatography gel in the presence of 1.25 M-ammonium sulphate (at which concentration most other peptides were not bound), and was eluted at about 700 mM-ammonium sulphate. Further purification of this fraction by reverse-phase h.p.l.c. on a C_{18} column gave a fraction containing only immunoreactive peptides, a major one having a molecular mass of 39 kDa and a minor one of slightly lower molecular mass. These peptides were sequenced together by automated Edman degradation (Table 1). The sequence of the first 20 amino acids of the major peptide is identical with residues 112–131 of the rat p65 sequence, which follow the potential trypsin-cleavage

Table 1. N-Terminal amino acid sequence of the 39 kDa soluble fragment derived from bovine p65 by proteolytic cleavage

About 32 pmol of peptide was loaded on to the sequencer, and the amino acid recoveries are corrected for background. In each case the amino acid quoted is that with the highest recovery in that cycle. The sequence of rat p65 (residues 112–131) is shown for comparison.

Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Amino acid	T	M	K	D	Q	A	L	K	D	D	D	A	E	T	G	L	T	D?	G	E
Yield (pmol)	28	19	17	14	28	26	28	11	8	10	6	17	9	9	15	7	5	2	7	8
Rat p65	T	M	K	D	Q	A	L	K	D	D	D	A	E	T	G	L	T	D	G	E

site at lysine-111. This sequence lies between the proposed transmembrane span and the first region, with sequence similarity with the C1 regulatory domain of protein kinase C (Fig. 6). The minor peptide was probably generated by cleavage of p65 at the next lysine (i.e. three residues closer to the C-terminus), as suggested by partial *N*-terminal sequencing.

The cleavage by trypsin of chromaffin granule p65 is consistent with results reported elsewhere (Fournier *et al.*, 1989a; Trifaró *et al.*, 1989) and with the sequence data for rat p65 (Perin *et al.*, 1990), and puts some restrictions on the location of the cgm67 epitope. The trypsin-cleavage sites shown in Fig. 6 are numbered according to their apparent distance (in kDa) from the *N*-terminus. The membrane-bound 45 kDa fragment (Fig. 4b, tracks 6–11) is produced by cleavage of p65 at site T22, and that of 28 kDa (Fig. 4b, tracks 10 and 11) by a further cleavage at T50. The soluble 39 kDa fragment (Figs. 4c and 4d, tracks 4–9) is produced by cleavage at T28, and the soluble 27 kDa fragment (Fig. 4d, tracks 9 and 10) is produced from this by removal of a 12 kDa fragment, from either its *N*-terminus (cleavage at T40) or its *C*-terminus (cleavage at T55), assuming that it is generated by a single cut. The cgm67 epitope must lie between T28 and T50; more precise location is not possible without further fragmentation and sequencing. In view of the large discrepancy between the molecular mass values determined by electrophoresis and by sequencing, the values quoted only give a rough idea of the relative sizes of these fragments. The extreme sensitivity of sites T22 and T28 to trypsin means that no membrane-bound tryptic fragment of molecular mass greater than 45 kDa is detected by cgm67.

The amino acid sequence data provide direct confirmation of the membrane orientation of p65. The sequence obtained is close to the start of the two regions similar in sequence to protein kinase C, which encompass most of the 39 kDa fragment. The calmodulin-binding domain lies within this fragment (Fig. 6), although it was not detected using the calmodulin overlay technique (Fournier *et al.*, 1989b; Trifaró *et al.*, 1989). The complete identity of the rat and bovine sequences for 20 amino

acids indicates a strong pressure for conservation in this region, which suggests some role in the function of p65.

Note added in proof (received 27 August 1991)

The results reported here are in agreement with those of a recently published study on the domain structure of p65 (Perin *et al.*, 1991).

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