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Alternative splicing gives rise to two forms of the p68 Ca^{2+}-binding protein

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The p68 Ca^{2+} and phospholipid binding protein of the lipocortin/calpactin family appears to exist as two forms. These may be resolved into a closely-spaced polypeptide doublet by SDS-PAGE. The cloning and sequencing of p68 revealed an apparent 18 nucleotide alternative splice sequence, which could account for this observation. We show here that an antiserum directed against a synthetic peptide corresponding to the region containing the splice sequence, recognises only the upper band of the p68 doublet by both immunoprecipitation and Western blotting., These results are consistent with alternative splicing being responsible for the generation of the two forms of p68.

p68; Ca^{2+}-binding protein; Lipocortin; Calpactin; Alternative splicing

1. INTRODUCTION

The lipocortin/calpactin family of Ca^{2+} and phospholipid binding proteins is now known to comprise at least 8 different members, as judged by cDNA sequence analysis. Although the functions of these proteins are not well understood (with the possible exception of synexin which exhibits Ca^{2+}-channel activity [1]), several diverse properties have been ascribed to them, such as inhibition of phospholipase A_2, inhibition of blood coagulation, actin-binding and collagen-binding (for reviews see [2,3]). The largest member of the family, namely p68 ($M_r = 68\,000$) (67 kDa calelectrin) was first identified in Ca^{2+}-dependent association with the detergent insoluble residue of B-lymphoblastoid plasma-membrane [4]. The protein invariably appears on denaturing gel electrophoresis as a closely-spaced polypeptide doublet, frequently with the upper band heavier than the lower. Molecular cloning revealed that p68 comprised 8 repeats of the 64 amino acid consensus sequence which both typifies and defines the lipocortin/calpactin family of proteins [5-7]. The other 7 related proteins, namely protein II, lipocortin I, calpactin I, endonexin II, lipocortin II, synexin and VAC-β, have molecular weights in the range 32 000-47 000, each containing 4 of the internally repeated sequence motifs. Although the proteins are closely related by virtue of their sequence similarities, each has a unique N-terminal sequence which it has been proposed may confer functional individuality [2].

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The sequence analysis of both human [5] and murine p68 [6] revealed an apparent alternative splice sequence of 18 nucleotides, encoding the hydrophobic hexapeptide Val-Ala-Ala-Glu-Ile-Leu (VAAEIL), close to the start of the seventh repeat. Alternative splicing of a single primary RNA transcript, which would give rise to two protein species differing by 6 amino acids, would provide both a convenient and plausible explanation for the characteristic p68 doublet observed on SDS-PAGE. To test the alternative splice theory as the mechanism for the origin of the two p68 species, rabbits were immunised with a synthetic DAQVAAEILEIC peptide conjugated to thyroglobulin, and the derivative antisera were used to investigate whether this sequence was unique to the upper band of the p68 doublet.

2. MATERIALS AND METHODS

2.1. *Antisera*

A rabbit anti-(human p68) immunoglobulin G (IgG) fraction (MC2) was prepared by standard methods [9] from a polyclonal antiserum of known specificity described previously [5], raised against purified denatured human tonsil p68. A control IgG fraction was prepared in the same way using pre-immune serum from the same rabbit.

A second antiserum (SS3) was generated by immunisation of rabbits with a synthetic DAQVAAEILEIC peptide (P) coupled via the C-terminal cysteine to thyroglobulin (P-TG). Since the actual proposed splice-sequence (VAAEIL) is small, it was considered that the inclusion of some flanking amino acids would improve the chances of creating an antigenic determinant. A peptide corresponding to the 10 N-terminal amino acids of human p68 coupled to thyroglobulin (N-TG), was used as a control in one experiment. Both peptides were kindly prepared by Dr J. Rothbard (ICRF). Rabbits were injected at multiple sites subcutaneously with 1 mg of P-TG in Freunds complete adjuvant. The rabbits were immunised again after 4 weeks using 1 mg P-TG in Freunds incomplete adjuvant, and bled 10 days later. Cycles

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of re-immunisation followed by test bleeds were continued as required, and serum from each bleed was stored aliquoted at −20°C. For affinity purification of SS3, an IgG fraction was first prepared from 50 ml antiserum [9], and this was passed over a column of peptide-bovine serum albumin conjugate (P-BSA), immobilised on CNBr-activated Sepharose 4B (Sigma Chemical Co. UK). Bound antibody was eluted with 3 M KSCN in 0.5 M NH_4OH, dialysed against ice-cold 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS), and concentrated to 1.85 mg/ml (total yield = 2 mg IgG from 50 ml serum). Aliquots were stored at −70°C.

2.2. *Cell culture and metabolic labelling*

The human T-leukaemia cell-line J6 was routinely maintained in RPMI 1640 medium containing 5% (v/v) foetal calf serum (FCS). For metabolic labelling, cells were first transferred to methionine-free RPMI 1640 medium containing 5% (v/v) dialysed FCS, for 1 h, to deplete the intracellular pool of methionine. This was followed by incubation in the same medium containing 50 μCi/ml [^{35}S]methionine (600 Ci/mmol, Amersham PLC, UK) for 4 h at 37°C. Prior to lysis, cells were washed twice with ice-cold PBS.

2.3. *Immunoprecipitation: SDS-PAGE and Western blotting*

Metabolically labelled cells were grown as described, and lysed (10^7 cells/ml) in 10 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl, 1% (v/v) Nonidet P-40, 5 mM diaminoethanetetraacetic acid (EDTA), 1 mg/ml BSA and 1 mM phenylmethylsulphonylfluoride (PMSF), for 2 min on ice. Lysates were centrifuged at 10000 × *g* for 10 min at 4°C to remove nuclei and membranes, and supernatants were retained for immunoprecipitation. Lysates were adjusted to give final concentrations of sodium dodecyl sulphate (SDS) as indicated in the figure legends.

Immunoprecipitation was achieved by the addition of MC2 (10 μg IgG fraction/10^6 cell equivalents) or SS3 (10 μg affinity-purified IgG/10^6 cell equivalents). Note: the quantities of MC2 and SS3 used in these experiments were determined empirically, and the relatively high amount of SS3 (affinity-purified IgG) required probably reflects the low affinity of this antibody compared to that of MC2 (total IgG). Samples were maintained on ice for 60 min prior to the addition of 50 μl 10% (w/v) protein A-Sepharose (Pharmacia, Sweden) in PBS containing 0.02% (w/v) NaN_3. Samples were mixed by rotation at 4°C for 30 min, briefly pelleted by centrifugation, washed twice with 1 ml ice-cold PBS, and finally resuspended in 80 mM Tris-HCl buffer pH 6.8 containing 4% (w/v) SDS, 4% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol and 0.02% (w/v) Bromophenol blue, heated at 95°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [10]. Preadsorption of antisera was performed by incubating aliquots of SS3 (10 μg IgG) with saturating quantities of either free peptide (5 μg) or peptide-conjugate (10 μg) on ice for 30 min.

Western blotting was performed by the method of Towbin et al. [11], and colour development of nitrocellulose filters was achieved using goat anti-(rabbit IgG) IgG coupled to horseradish peroxidase (Tago Inc. USA) with 4-chloronaphthol as colour reagent.

3. RESULTS

In order to determine the specificity of antiserum SS3, a combination of Western blotting and immunoprecipitation experiments were performed, in conjunction with antiserum MC2. The model being tested is shown in fig.1, which illustrates schematically the proposed structures of the two bands of the p68 doublet. The results in fig.2 (panels A and B), show that both antisera demonstrated SDS-dependent immunoprecipitation of [^{35}S]methionine labelled p68, from J6 cells. MC2 was found to be capable of immunoprecipitating p68 at SDS levels ranging from 0.1% to 1.0%, whereas SS3 was most effective at 0.2% to 0.5%. Significantly, SS3 which was raised against the peptide corresponding to the p68 alternate splice sequence, immunoprecipitated a single polypeptide at 68 kDa, whereas MC2 which was raised against the whole protein, immunoprecipitated the characteristic 68 kDa doublet. Non-specific precipitation of other bands was observed, although for MC2 these were also represented in the pre-immune serum tracks (fig.2, panel A). Considerably more background bands were observed with SS3 (fig.2, panel B), although with increasing SDS concentration, these diminished whereas only the 68 kDa band exhibited increased intensity.

Fig.2, panel C shows that the 68 kDa band immunoprecipitated with SS3 migrated with the same mobility as the upper band of the MC2 p68 doublet.

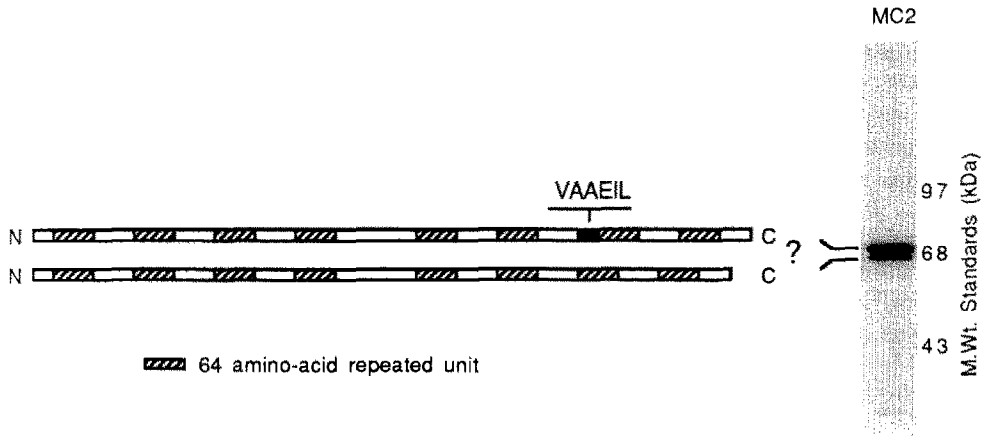

Fig.1. Schematic model for the proposed structures of the two forms of p68. Both bands of the p68 doublet are illustrated, each containing the 8 repeated units. The position of the alternative splice sequence at the start of the seventh repeat is also indicated, but only in the upper band. The autoradiograph on the right shows [^{35}S]methionine-labelled p68 immunoprecipitated with MC2 in the presence of 0.5% SDS, from a lysate of T-leukaemia J6 cells.

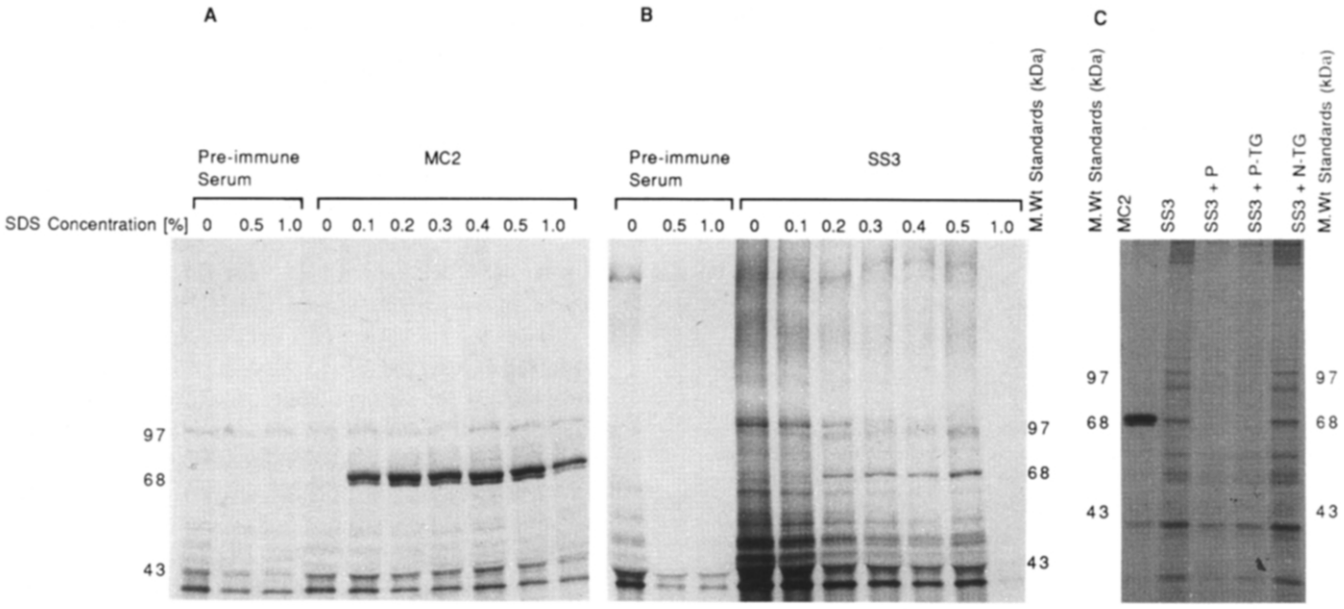

Fig.2. SDS-dependent immunoprecipitation of p68. Panels A and B show autoradiographs of [^{35}S]methionine-labelled p68 immunoprecipitated with MC2 and SS3, respectively, from J6 lysates, in the presence of increasing levels of SDS. Panel C shows the effect of preadsorption of SS3 with either P, P-TG or N-TG. The positions of molecular mass protein standards are also indicated. These were phosphorylase B (97 000), BSA (68 000) and ovalbumin (43 000).

Preadsorption of SS3 with either free peptide (SS3 + P) or peptide-conjugate (SS3 + P-TG) abrogated immunoprecipitation of the 68 kDa band, whereas preadsorption with a peptide corresponding to the p68 N-terminus (SS3 + N-TG) had no effect. These observations indicate that the antiserum SS3 recognises a polypeptide which co-migrates on SDS-PAGE with the upper band of the p68 doublet, and that the polypeptide shares an antigenic determinant(s) with the peptide DAQVAAEILEIC. However, the upper band of the p68 doublet immunoprecipitated by MC2, was more intense on autoradiography than the single 68 kDa band produced by SS3 (fig.2, panel C). This raised the possibility that the apparent absence of the p68 lower band in the SS3 precipitates could simply be due to a lack of sensitivity of the technique.

To confirm that the polypeptides recognised by these antisera were identical, a system of immunoprecipitation coupled to Western blotting was used, whereby the immunoprecipitated material generated by one antiserum was tested against the other by immunoblotting. The results in fig.3 clearly show that if p68 is immunoprecipitated by MC2 (in the presence of 0.5% SDS), SS3 identifies only the upper band on Western blotting (panel A, lane 2). The alternate use of MC2 in the blotting stage (panel B, lane 2), confirms that both polypeptides were immunoprecipitated and successfully transferred to nitrocellulose. Conversely, when SS3 was used to immunoprecipitate p68 (in the presence of 0.3% SDS), MC2 identified only the upper band on Western blotting (panel B, lane 3). As expected, alternate use of SS3 in the blotting stage (panel A, lane 3) confirmed the presence of only the upper band. These findings were substantiated by Western blot analysis of both a J6 whole cell lysate and purified p68 (generously provided by H. Edwards and W. Verbi, ICRF). In both cases, SS3 recognised only a single band at 68 kDa (panel A,

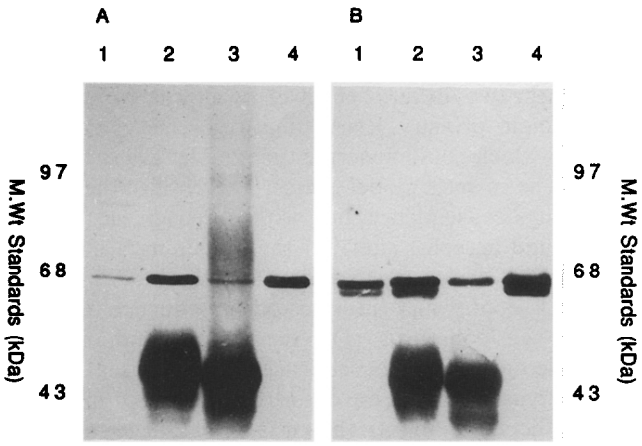

Fig.3. Western blot analysis of SS3 and MC2. Panel A was immunoblotted with SS3 and panel B with MC2. (Lane 1) J6 whole cell lysate; (lane 2) MC2 immunoprecipitate; (lane 3) SS3 immunoprecipitate; (lane 4) purified placental p68. The positions of prestained molecular mass markers are also indicated (see legend to fig.2). The heavily staining bands at approximately 50 kDa, in lanes 2 and 3 in both panels are rabbit IgG heavy chains, arising from the immunoprecipitation stage, and directly detected by the second antibody (goat anti-(rabbit IgG) IgG coupled to horseradish peroxidase) during the blotting procedure.

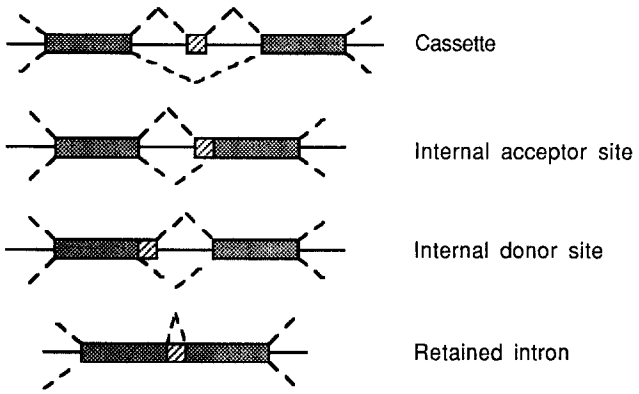

Fig.4. Mechanisms of alternative splicing. The figure shows diagramatically the possible mechanisms of splicing for p68. The constitutively expressed exons are shown as hatched boxes, and the alternatively spliced sequence as a striped box. The dotted lines indicate possible splicing pathways (adapted from Breitbart et al. [8]).

lanes 1 and 4, respectively), whereas MC2 recognised both bands (panel B, lanes 1 and 4, respectively). These observations demonstrate that MC2 and SS3 cross-react immunologically with the same polypeptide, namely the upper band of the p68 doublet, but that MC2 also cross-reacts with the lower band.

4. DISCUSSION

In these studies, using an anti-(peptide) serum, we have provided compelling evidence that the VAAEIL hexapeptide sequence is unique to the upper band of the p68 doublet, as revealed by SDS-PAGE. From these results, supported by the observation that single gene loci were identified during the chromosomal assignments of both the human and murine p68 genes (to chromosomes 5 and 11, respectively) [13], it is concluded that the two forms of p68 arise by alternative splicing of a single primary RNA transcript. The 4 splicing models which could generate the doublet are shown in fig.4. The cassette model is most likely, since the alternative splice sequence lies within recognised splice donor and acceptor sites. Of the remaining 3 models, the internal donor site is more probable, in view of its stronger conformity in nucleotide sequence to the donor site consensus. On the other hand, if the 18-nucleotide p68 splice sequence is an individual exon, rather than a retained intron or the product of internal exon splicing (see fig.4), then it is amongst the smallest reported (the cardiac troponin T gene has a 6-nucleotide exon [12]). The possibility remains, albeit unlikely, that the two forms of p68 are encoded by separate genes, differing only with respect to the sequence of 18 nucleotides identified during cloning, lying within close proximity on the same chromosome, thereby defying resolution by in situ hybridisation.

Alternative splicing is a well-recognised characteristic of eukaryotic genes, providing a sophisticated means of generating more than one protein product from a single gene [8]. Although many genes are now known to generate protein diversity through alternative splicing, p68 is the only member of the lipocortin/calpactin family to have been identified with this phenomenon. We have previously proposed that the p68 gene may have arisen by duplication of a gene encoding a 4 repeat protein [2]. In this case, since the N-terminal half of p68 resembles the 4 repeat proteins more closely than the C-terminal half, it seems likely that the alternative splice sequence arose during or after the proposed gene duplication event. It is not yet known whether alternative splicing of p68 is functionally significant, but the production of antibodies which can discriminate between the two forms, will clearly be useful in addressing this question.

Added note: A transformation-related change in the alternative splicing of p68 has recently been observed. In Swiss 3T3 fibroblasts, progression in culture from a normal to a transformed phenotype was accompanied by a change in the ratio of p68 upper band to lower band, from 2:1 to 0.8:1, as judged by densitometric scanning of autoradiographs of [^{35}S]methionine-labelled polypeptides, immunoprecipitated from metabolically labelled cells and separated by SDS-PAGE.

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