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A discontinuous epitope on p36, the major substrate of src tyrosine-protein-kinase, brings the phosphorylation site into the neighbourhood of a consensus sequence for Ca^{2+}/lipid-binding proteins

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Previous models of p36 based on proteolytic fragments describe the tail and core as two noninteracting domains. However, the monoclonal antibody H28 recognizes a discontinuous epitope, which covers the peptide segments around Ser 25 in the tail and around Glu 65 in the core of porcine p36. Thus, the phosphorylatable Tyr 23 is much closer to the first consensus sequence (residues 46–62) of Ca^{2+}/lipid-binding proteins than previously thought. This apposition is in line with biochemical experiments indicating an influence of core ligands on tyrosine phosphorylation and an enhanced Ca^{2+} requirement of the modified p36 in phospholipid binding.

Calpactin; Discontinuous epitope; Lipocortin; src kinase; Tyrosine phosphate

1. INTRODUCTION

p36, the major cytoplasmic target of src-encoded tyrosine-protein-kinase, is usually isolated as a heterotetramer with a p11 polypeptide [1]. The p11 dimer forces p36 into the heterotetramer complex and modulates some of the binding properties shown by the monomeric p36 [2–4]. p36 displays the sequence principle characteristic for a new class of Ca^{2+}/lipid-binding proteins [5]. The large core domain spans four homologous segments and displays Ca^{2+} as well as Ca^{2+}-dependent lipid binding [6,7]. The short amino-terminal tail harbors the p11-binding site and bears the tyrosine phosphorylation site as well as a site recognized by kinase C [7–11]. As tail and core domains can be separated by limited proteolysis [7,9], current models assume that the tails of two p36 molecules anchor to the p11 dimer without further contacts [12]. This view does not allow for a communication between the two domains of p36. It does not explain why p36 phosphorylated at Tyr 23 has an enhanced Ca^{2+} requirement in lipid binding [4] and why tyrosine phosphorylation is enhanced in vitro by core ligands [13,14]. The characterization of the discontinuous H28 epitope given below provides for an unexpected proximity of the tail domain and the core, which allows a direct communication.

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2. MATERIALS AND METHODS

Porcine, bovine and chicken p36 were purified from intestinal epithelium essentially as in [1,15]. Human p36 was isolated from placenta using the same protocol. A crude murine p36 fraction was obtained by extracting intestinal epithelium first with Triton X-100 and Ca^{2+} and then with EGTA [1]. Chymotryptic, tryptic and V8 protease cores of porcine p36 were obtained by mild proteolysis [15]. The in situ proteolysis derivative p34 [7], murine monoclonal antibody H28 [16] and rabbit antibodies to porcine p36 [1] have been described. Automated gas-phase sequencing (Applied Biosystems model 470A) was performed on the chymotryptic cores of porcine and chicken p36 and on N-terminal peptides. Western blots were usually treated with H28 and the second antibody at 37°C to conform with the previous immunofluorescence microscopical

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data [16]. Competitive immunoblots were at room temperature. For carboxylate modification porcine p36 was treated with 1 M glycine methyl ester and 30 mM EDC [1-ethyl-3-(*N,N*-dimethyl)aminopropylcarbodiimide] in 0.1 M Mes (pH 5.4). The reaction was stopped after 30 and 90 min with 1 M sodium acetate (pH 5.4). Subsequent treatment with hydroxylamine was used to ensure reversion of a possible tyrosine modification. p36 was cleaved with CNBr using standard procedures. Fragment f_1 was separated from other peptides by gel chromatography in 9 M urea, 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 3 mM DTT on an S200 column (Pharmacia). The fragment was renatured [2] by dialysis against 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM NaN_3, 0.5 mM EGTA, 2 mM DTT. The blocked N-terminal end and the amino acid composition identified fragment f_1 as spanning residues 1–117.

3. RESULTS

H28 antibody reacts strongly in Western blots with porcine, chicken and bovine p36 purified from intestinal epithelium (fig.1C). No reaction was observed with human p36 purified from placenta or the murine p36 present in an extract of intestinal cells. In order to map the epitope several proteolytic derivatives of porcine p36, which lacked increasing numbers of N-terminal residues, were used in Western blos (fig.1A). While the V8 protease core starting at Gly 14 harbors the H28 epitope, no antibody reactivity was found with the tryptic and chymotryptic cores, which begin with Ala 28 and Thr 30, respectively. A proteolysis product of molecular mass 34 kDa obtained as a by-product during protein I purification [7] also bound H28. Automated sequencing of this material revealed an equimolar mixture of core molecules starting at Ser 25 and Ala 28, respectively. In standard proteolysis experiments [7] H28 inhibited the cleavage of porcine p36 observed by chymotrypsin or trypsin.

The results on the different core derivatives indicate that the region starting at Ser 25 forms part of the H28 antibody-binding site. If this site forms a continuous linear epitope, the sequences of p36 from different species should explain the limited cross-reactivity of H28 observed (fig.1) by obvious amino acid replacements. However, the H28-reactive bovine protein and the non-reactive human protein show no amino acid replacement between residues 1 and 64 ([17,18] see fig.2). Thus, the epitope must involve an additional region. In the further characterization of this discontinuous (conformational) epitope, we were greatly helped by the currently available p36 sequences. Since human and bovine p36 differ by only 5 replacements, we analyzed additional fragments of porcine p36 for H28 binding. Reactivity was retained on residues 1–212 obtained by cleavage at the single tryptophan [7]. More importantly, immobilized H28 absorbed from the total mixture of CNBr fragments the large N-terminal fragment f_1, which spans residues 1–117 (figs 1B,3A). Within this region human and bovine p36 differ only at position 65. Therefore, we extended our previous sequence data [7] on porcine p36. The chymotryptic core was sequenced until Gln 68. As shown in fig.2 the H28-positive proteins from cow and pig show identical sequences for residues 21–68, while the immunologically distinct human protein shows a single amino acid exchange, i.e. Glu 65 is replaced by Ala. To consolidate this deduction, we determined the N-terminal 69 residues of chicken

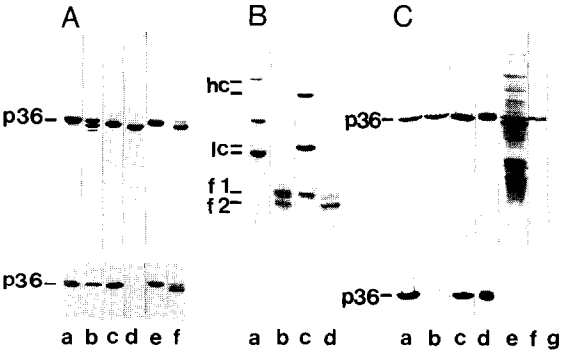

Fig.1. H28 reactivity of porcine p36 requires part of the N-terminal tail domain. The upper and lower part of panel A show gel patterns and corresponding Western blots of porcine p36 (lanes a,e), the p34 in situ proteolysis product (lane f) and two purified core domains. Note the loss of the epitope in the chymotryptic core (lane d) and its presence in the V8 protease core (lane c). In mildly trypsinized p36 (lane b), reactivity is restricted to undigested material (top band) and lost in the core domains starting either at Ala 28 (middle band) or at a yet unidentified residue (bottom band in upper part). Immunoprecipitations with H28 are shown in B (lanes a,c). hc and lc designate the IgG heavy and light chains, respectively. Experiments were performed on an EGTA extract from bovine MDCK cells (lane a) or with CNBr-treated porcine p36 (lane c). Lane b shows the larger fragments f_1 and f_2 prior to immunoprecipitation. Note that H28 absorbs specifically the 12 kDa N-terminal fragment f_1 (lane c), while f_2 remains non-bound (slot d). The limited cross-species reactivity of H28 is shown in panel C by gel patterns (lanes a–d, upper part) and corresponding Western blots (lower part) of porcine (lanes a), human (lanes b), chicken (lanes c) and bovine p36 (lanes d). In a crude extract of mouse intestinal cells (lane e) a polyclonal antibody detects murine p36 (lane f), but H28 does not (lane g).

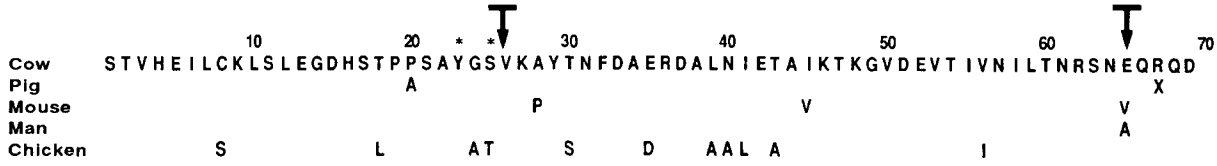

Fig.2. The N-terminal sequences of p36 from 5 different species. Sequences for murine, bovine and human p36 have been deduced from cDNA clones [5,17,18]. Protein sequences of porcine and chicken p36 are based on automated sequencing of the core domains and characterization of the N-terminal peptides. Only the variant positions vs bovine p36 are given. Note the presence of a glutamic acid residue at position 65 in the H28-positive proteins from pig, cow and chicken and the change to alanine (human) and valine (mouse) in the non-reactive proteins. X denotes an as yet unidentified residue in porcine p36. Over the region shown, the non-reactive human p36 differs only at position 65 from the reactive bovine p36. Arrows indicate the two peptide segments proposed for the discontinuous epitope. The horizontal line indicates the consensus sequence of Ca^{2+}/lipid-binding proteins. Identified phosphorylation sites are designated by asterisks (see text).

p36, which also harbors the H28 epitope (fig.1C). Although the avian protein differs by several conservative replacements from the mammalian proteins, it displays Glu 65 as do the other two H28-reactive proteins (fig.2). Three replacements in chicken p36 locate directly in or close to the first part of the epitope deduced above. They involve the substitution of an alanine for a glycine (position 24), a threonine for a serine (position 25) and a serine for a threonine (position 30). All three substitutions are highly conservative replacements. The amino acid requirements of the H28 epitope also explain its absence in murine p36 (fig.1C). As in the non-reactive human p36, Glu 65 is replaced by an uncharged residue. This is a valine for murine p36 [5] and an alanine for the human protein [18]. Given the importance of Glu 65 for H28 reactivity porcine p36 was treated with glycine methyl ester and EDC to esterify the acidic residues at the protein surface. Fig.3A shows that the chemical modification abolished antigenicity in a subsequent blot. In addition, esterified p36 was not precipitated by H28 antibody (not shown). After a similar EDC treatment of porcine p36 complexed with H28 antibody reactivity of p36 was preserved when the separated polypeptides were probed with H28 on an immunoblot (fig.3A).

To compare the relative affinities of the different proteins for H28 immobilized porcine p36 was probed with solutions containing a constant amount of antibody and increasing concentrations of a competing antigen (fig.3B). In this assay porcine p36 showed at least a 200-fold higher affinity for H28 than the human protein. The N-terminal CNBr fragment f_1 of porcine p36 showed about 13-fold lower affinity than the intact protein. The decreased affinity of the fragment could be due either to imperfect refolding or to the presence of an as yet unidentified third peptide segment in the H28 epitope.

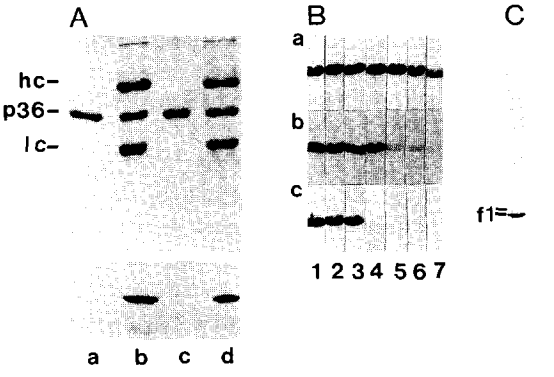

Fig.3. (A) Gels (upper part) and corresponding blots (lower part) of porcine p36 treated for 15 min (lanes a,b) or 90 min (lanes c,d) with EDC in the absence (lanes a,c) or presence of H28 antibody (lanes b,d). Note the loss of H28 reactivity upon EDC treatment in the subsequent blot (lanes a,c, lower part) and the protection of the epitope in the presence of H28 (lanes c,d, lower part). In panel B a constant amount of porcine p36 was blotted on nitrocellulose. Blots were then treated with an H28 antibody concentration of approx. 5 nM in the presence of added human p36 (part a), porcine p36 CNBr fragment f_1 (part b) and porcine p36 (part c). Blots were subsequently washed and treated with a peroxidase-labelled second antibody. In lane 1 the concentration of human and porcine antigen was 3 μM, that of the f_1 fragment being 2.5 μM. Lanes 2–7 show the results when the competing antigen was diluted by factors of 3, 6.5, 30, 300, 3000 and 300000, respectively. Note the very poor competition by human p36 in slots 6 and 7 of a vs the good competition with porcine p36 (slots 4–7 of c) and the competition by the CNBr fragment. Panel C shows a gel of the porcine p36 N-terminal CNBr fragment (f_1), purified by gel filtration, which was used for the competition in panel B (part b).

4. DISCUSSION

Several studies using proteins of known three-dimensional structure have shown that the majority of antibodies elicited involve discontinuous epitopes. These consist of several peptide segments brought together by the correct folding. Identification of discontinuous epitopes is often helped by the limited cross-species reactivity of monoclonal antibodies due to known amino acid replacements arising in a series of homologous proteins (review [19]). In line with this concept, the H28 epitope provides the first information on the folding of p36. The epitope combines the peptide segment around Ser 25, located in the tail domain, with the peptide segment around Glu 65 located in the core domain. Thus, previous models describing tail and core as two non-interacting domains [12] are clearly oversimplifications.

An interesting aspect of the H28 epitope concerns the potential influence of tyrosine phosphorylation on established properties of p36. Tyr 23 and Ser 25, the targets of src-kinase and kinase C, respectively [9–11], are situated close to or within an epitope, which also covers Glu 65. Interestingly, the latter residue follows the Geisow consensus sequence [20] in the first core segment (residues 46–62, fig.2). Related sequences are present in a similar relative location in all 4 core segments of the Ca^{2+}/lipid-binding proteins [5,17,18]. Thus, the phosphorylation sites are in a much closer proximity with a unique consensus sequence, believed to be involved in Ca^{2+} and/or phospholipid binding [20], than previously anticipated. Interestingly, tyrosine-phosphorylated p36 has a higher Ca^{2+} requirement for lipid binding than unmodified p36 [4] and tyrosine phosphorylation of p36 by src-kinase is markedly enhanced by Ca^{2+} and phospholipids [13]. While these results cannot be explained by previous models, they fit the view developed by the characterization of the discontinuous H28 epitope. The N-terminal 12 residues of p36 form as an α-helix the major binding site of p36 for the p11 molecule [15]. In line with this restriction of the major p11-binding site to the N-terminal half of the tail, H28 antibody binds to p36 as well as to p36 complexed with p11 (not shown). As the p36/p11 heterotetramer has a lower Ca^{2+} requirement for lipid binding than p36 [3,4], we speculate that the sequence around residue 25 could bring the p11-binding site with its p11 ligand into apposition with the Ca^{2+}/lipid-binding site.

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