Binding sites for calcium, lipid and p11 on p36, the substrate of retroviral tyrosine-specific protein kinases

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Biochemical and partial sequence data reveal the two-domain structure of p36. A loose structure of some 30 residues at the amino-terminus contains the phosphorylatable tyrosine and the binding site for the p11 regulatory chain. The following p33 domain retains the lipid-binding site as well as the Ca^{2+} site which influences the spectral properties of the single tryptophan and one tyrosine. The combined sequence data covering about 25% of the molecule identify p36 as a unique polypeptide.

Ca^{2+} (Intestine) Lipid Membrane protein Tyrosine phosphate

1. INTRODUCTION

Certain oncogenic viruses encode tyrosine-specific protein kinases and related enzymatic activities are known to be associated with receptors for some growth factors (review [1]). A major cellular substrate of fibroblasts transformed by retroviruses is a 36 kDa protein, called p36. Although it has been known for several years, neither the function of p36 nor the possible importance of its phosphorylation has emerged. With the realization that p36 can be purified in large amounts from intestinal epithelium [2], molecular properties could be established which may finally help in understanding the role of this protein, which is considered as membrane and/or cytoskeletal element [2,3].

p36 is a Ca^{2+}-binding protein of moderate affinity [2,3] and this binding seems to be enhanced by phosphatidylserine (PS) but not by phosphatidylcholine (PC) [4]. Ca^{2+} binding induces a large conformational change exposing the single tryptophan and at least one tyrosine to a more aqueous environment [3]. This molecular change also leads to in vitro binding to F-actin and spectrin, the physiological importance of which is not understood [2,3]. p36 can exist either as a monomer or as a complex (protein I) containing two copies of p36 and a dimer of a unique p11 polypeptide [2,3]. p11 is related by amino acid sequence to S-100 [5,6], a protein with unknown function. Here we explore the binding sites of p36 and characterize the protein by further biochemical properties as well as by partial amino acid sequences.

2. MATERIALS AND METHODS

Porcine protein I and subunits p36 and p11 were obtained from intestinal epithelium [2,3] using the modification given in [5]. Ca^{2+} ultraviolet difference spectra were recorded as described [3]. In vitro proteolysis of protein I and p36 was performed at 20°C in 20 mM imidazole-HCl (pH 7.4), 90 mM NaCl, 0.5 mM DTT, 2 mM NaN_3 using either Ca^{2+} or EGTA at 1 mM. Proteins were present at about 1 mg/ml and enzymes at 0.005 mg/ml. Proteolysis was stopped by either the addition of the hot sample buffer used for gel electrophoresis or phenylmethylsulfonyl fluoride (PMSF) to 2.5 mM. For p33 isolation, p36 was treated with chymotrypsin for 25 min in 0.1 M

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NH_4HCO_3. After addition of PMSF gel filtration was executed in 0.1 M NH_4HCO_3 on Sephadex G-100. Alternatively the digest was lyophilized and p33 purified by HPLC. Lipid binding was carried out in 20 mM imidazole-HCl (pH 7.4), 80 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT using either 1 mM EGTA or 0.1 mM $CaCl_2$ and PS-containing liposomes (1 mg/ml). After centrifugation (20 min, 300000 × *g*) corresponding aliquots of supernatant and pellets were characterized by SDS-polyacrylamide gel electrophoresis. Molecular masses of derivatives were deduced from gel filtration [2,3]. For fast screening of digestion conditions, gel filtration was performed by FPLC (Pharmacia) in EGTA-containing Tris buffer on a Superose S12 column. Cleavage at tryptophan with BNPS-skatole was performed on p36 previously carboxymethylated with iodoacetic acid in 8 M urea using standard conditions [7].

3. RESULTS

During purification of protein I [2,3] a proteolytic derivative of p36 elutes earlier on the carboxymethylcellulose column. This derivative, p34, is recognized by antibodies to p36 in immunoblots and is a monomer in gel filtration. Gel electrophoresis shows that p34 lacks the p11 subunit. In the search for a similar derivative available in large amounts, in vitro proteolysis of protein I and p36 was studied (fig.1). Trypsin yielded a p33 which was time-dependently digested further into

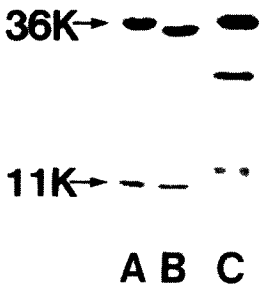

Fig.1. Chymotryptic cleavage of protein I (lane A) provides a stable p33 (lane B) leaving p11 intact. Gel pattern of p36 after treatment with BNPS-skatole in lane C.

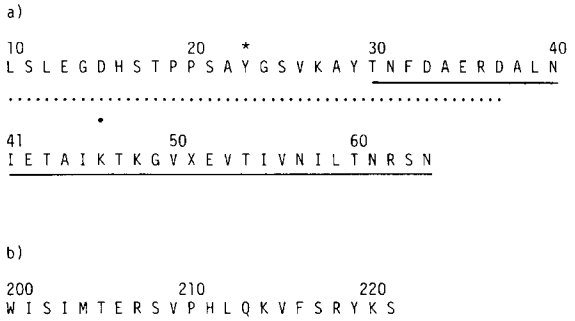

Fig.2. Sequence results on fragments of porcine p36. (a) The chymotryptic p33 derivative starts at residue 30 and the determined sequence continues to residue 64. X indicates a not yet identified residue. This sequence of 35 residues (underlined) overlaps by 8 residues with the previously reported 28 residues (dotted) of bovine p36 [6]. (b) The 21 residues following the single tryptophan (W) of porcine p36. Residue numbers are estimated from the size of the fragments obtained by BNPS-skatole cleavage (see text). Note that the single tryptophan and the phosphorylatable tyrosine (indicated by an asterisk) are widely separated in the linear sequence.

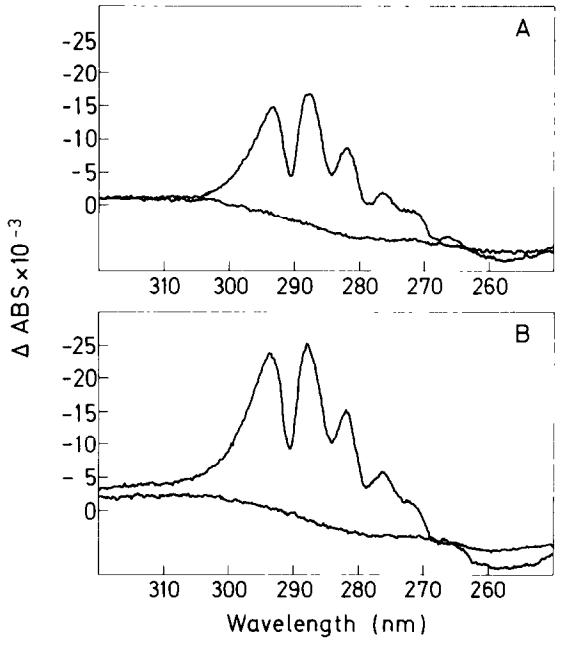

Fig.3. Calcium-induced difference spectra of p36 (A) and its proteolytic p33 derivative (B). Protein concentrations were 0.8 (A) and 1.1 mg/ml (B), respectively.

smaller fragments. As this process was enhanced by EGTA a Ca^{2+}-binding site was indicated for p33. Chymotrypsin, however, provided independently of calcium or EGTA a p33 of high stability. This derivative was purified. When in a digest of protein I, p36 was completely converted to p33, the p11 was not changed and could again form protein I once p36 was added.

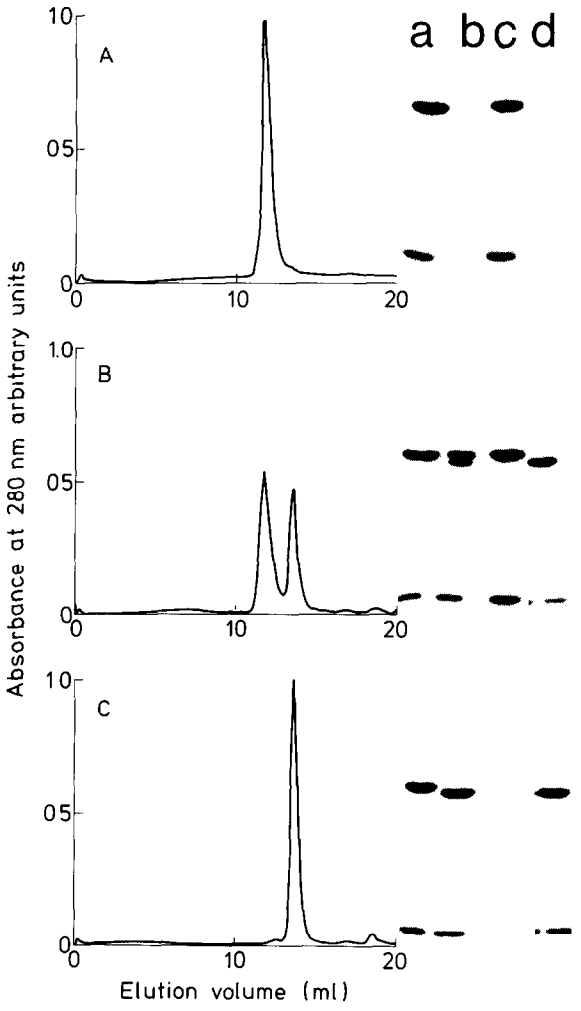

Fig.4. Gel filtration on Superose S12 monitored by FPLC. Protein I (A) was treated with chymotrypsin for 1 min (B) and 18 min (C) before addition of PMSF. Control experiments showed the elution positions of protein I (11.7 ml), p36 and p33 (13.7 ml) and p11 dimer (13.8 ml). Gels shown in B and C are for protein I (lanes a; control), chymotryptic digest before gel filtration (lanes b) and the fractions corresponding to 11.7 and 13.7 ml, respectively (lanes c,d).

While p36 has a blocked N-terminus, a run of p33 on the gas-phase sequenator yielded a unique sequence of 35 residues (fig.2). Of the proteolyzed 3 kDa domain two smaller peptides were isolated and characterized but the peptide carrying the blocking group, possibly myristic acid [8], has not yet been recovered from the HPLC. p33 as well as p34 retain the Ca^{2+}-induced ultraviolet difference spectrum typical for p36 (fig.3). This is characterized by the exposure of the sole tryptophan and at least one tyrosine to a more aqueous environment in the presence of the ligand [3]. The relative position of the tryptophan in the p36 molecule was mapped. Cleavage at tryptophan of *S*-carboxymethylated p36 using BNPS-skatole provided two fragments of p36 (fig.1). While the larger 23 kDa fragment retains the blocked N-terminus of p36, the smaller 13 kDa fragment could be sequenced for 21 residues (fig.2). Thus the sole tryptophan is about 200 residues removed from the N-terminus of p36. The two sequence stretches established (fig.2) account for about 25% of the p36 sequence and were unique when entered into the Protein Sequence Data Bank of the National Biomedical Research Foundation.

Purified p33 as well as p34 behaved as monomers in gel filtration. Their elution profile

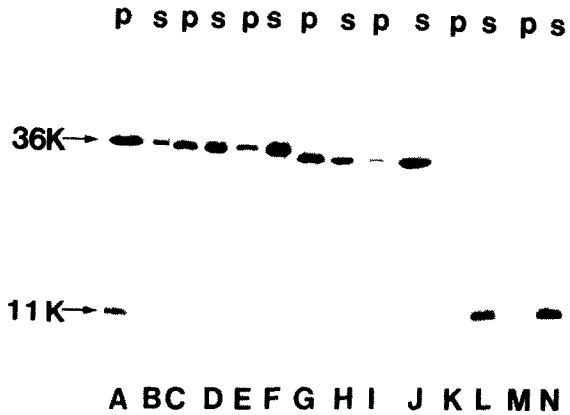

Fig.5. Binding to PS liposomes monitored by cosedimentation and gel electrophoresis. p, pellets; s, supernatants. Results are for protein I (lanes A,B), p36 (lanes C–F), p33 (lanes G–J) and p11 (lanes K–N). Assays were in Ca^{2+} (lanes A–D,G,H,K,L) or in EGTA (other lanes). Binding of p36 and p33 is Ca^{2+} dependent but reduced in comparison to protein I. p11 shows only background binding in the presence of Ca^{2+}.

did not shift when mixed with p11. Control experiments with p36 showed as before [3] reconstitution to the tetrameric complex containing two copies each of p36 and p11. Thus the proteolytic removal of about 30 residues from the N-terminus of p36 results in a p33 domain unable to bind p11 (fig.4). p33 also retains the Ca^{2+}-dependent interaction with F-actin (not shown). Liposomes of PS bound protein I in the presence of Ca^{2+} (see also [4]). This binding (fig.5), although somewhat weaker, is retained in monomeric p36 and p33, while p11 failed to bind above background levels.

4. DISCUSSION

We have delineated two structural domains of p36. The amino-terminal 30 residues are necessary to bind p11, which induces the complex (protein I) of two copies each of p36 and p11. The carboxy-terminal p33 domain carries the Ca^{2+} site influencing the environment of at least one tyrosine and the sole tryptophan now mapped to position 200 (fig.2). This domain also harbors the lipid-binding site. The partial amino acid sequences accumulated during the characterization of the domains cover about 25% of the molecule and identify p36 as a unique protein.

Under in vitro conditions tyrosine phosphorylation is stimulated by Ca^{2+} [3,5,9]. Thus at least two tyrosines are influenced by Ca^{2+}. The phosphorylatable tyrosine which is mapped in the amino-terminal region, and a second tyrosine, which is located in p33. The latter tyrosine changes its spectral properties together with the tryptophan. The Ca^{2+}-induced enhancement of phosphorylation most likely arises from a conformational influence of the p33 domain on the amino-terminal domain. That monomeric p36 seems a better substrate than the complex with p11 [9] is in line with a binding site for p11 in the amino-terminal domain. When we used secondary structure prediction rules [10] the tyrosine phosphate is present within a stretch containing several β-turns (residues 6–17) while the cleavage point for chymotrypsin precedes a short, putatively α-helical element (residues 24–32).

Myristylation of p36 in fibroblasts was not detected in [11] although a more recent report [8] has raised this possibility. The amino-terminus of p36 is blocked ([6] and above) but the nature of the blocking group is unknown. It will become available once the very amino-terminal peptide can be isolated from the chymotryptic digest which leads to p33. However, even if it was a fatty acid our results show that the lipid-binding site of p36 resides in the carboxy-terminal p33 domain. As shown for the Ca^{2+}-induced binding of protein I and p36 to F-actin and spectrin [2,3], binding to PS was more effective with protein I, which contains two p36 molecules, than with monomeric p36 or p33. Turbidity measurements implicate protein I in Ca^{2+}-dependent aggregation of PS liposomes. Future experiments should characterize the sequences involved in lipid and Ca^{2+}-binding in order to understand the interaction between the two sites.

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