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HOMOLOGY BETWEEN THE SUBUNITS OF S100 AND A 10kDa* POLYPEPTIDE ASSOCIATED WITH p36 OF PIG LYMPHOCYTES

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The polypeptide of about 10,000-Mr which is associated with the p36 polypeptide of pig mesenteric lymph node lymphocytes has a N-terminal 51 amino acid sequence that is 56 and 42% homologous with the N-terminii of the α and β subunits respectively of S100.

The p36 polypeptide was originally identified in transformed chicken embryo fibroblasts as the major substrate for the tyrosine-specific phosphorylation by pp60src kinase [1,2]. Subsequently, p36 was detected in a variety of normal tissues and cell lines of various species [3,4], although it's level of expression varied markedly in different cell types. Although p36 was not revealed in normal lymphocytes by immunohistochemical procedures, it was detected by Western blotting of the Nonidet P40-insoluble residue of purified lymphocyte plasma membrane [5]. Two recent reports [6,7] indicate that the p36 polypeptide of the brush border membrane of pig intestinal epithelium and of chicken embryo fibroblasts is specifically associated with a lower molecular weight polypeptide of about 10,000 and 6,000-Mr† respectively. The composite molecules, namely "protein 1" in the pig and "34K-L" in the chicken, were described as having molecular weights of from 82,000 to 88,000 and 60,000 to 70,000 respectively.

* This designation, which is based on a previous assignment, will be used in this paper even though the polypeptide had a Mr of about 9,000 and 6,000,as judged by gel filtration in guanidine HCl and SDS/PAGE respectively.

†Abbreviations : Mr, molecular weight; SDS/PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis; DTT, dithiothreitol; ICBP, intestinal Ca^{2+}-binding protein.

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This paper describes the isolation of a larger molecular weight form of p36 from the EGTA-extract of the Nonidet P40-insoluble residue of the plasma membrane fraction of pig mesenteric lymph node lymphocytes. The purified protein comprised, apart from p36, a polypeptide of about 10,000-Mr. Amino acid sequence analysis of the smaller polypeptide indicated that its N-terminal sequence resembled, in particular, that of the α subunit of the S100 protein.

MATERIALS AND METHODS

Pig mesenteric lymph nodes ("gut fat") and small intestine were obtained from a local abbatoir (British Beef, Watford, Herts.). Lymphocyte microsomes and plasma membrane, prepared as previously described [8], were extracted with 1% Nonidet P40 and the insoluble residue was re-extracted with 1mM EGTA in 1% Nonidet P40 to yield a soluble fraction [9]. The latter fraction has been shown previously by SDS/PAGE to comprise principally polypeptides of about 68,000, 36,000 and 32,000-Mr. Brush border membranes of pig small intestine were prepared and extracted with EGTA as described by Gerke and Weber [7]. The 36,000-Mr polypeptide of both EGTA extracts bound antibodies against chicken fibroblast p36, as revealed by Western blotting [5,7].

The larger molecular weight form of p36 was separated from each EGTA extract by ion-exchange FPLC (Pharmacia, Uppsala, Sweden). Briefly, the extracts were dialysed against 20mM triethanolamine HCl buffer, pH 7.4, containing 2mM EGTA and 1mM DTT and were then passed through a Mono Q column. The unretarded fraction, after dialysis against 50mM Na malonate buffer, pH 5.6, 2mM EGTA, 1mM DTT was eluted from a Mono S column with a gradient of NaCl in the same buffer. p36 associated with a polypeptide of about 10,000-Mr was eluted by 0.26M NaCl.

The p36 and 10,000-Mr polypeptides were separated either by preparative SDS/PAGE, as previously described [10], by using a 170x240x2mm 12% polyacrylamide slab gel, or by elution from a Superose 12 column in 6M guanidine HCl, 0.25M Tris HCl buffer pH 7.5, after reduction in 0.5ml of 10mM DTT, 6M guanidine HCl, 0.25M Tris HCl, pH 7.5 for 2hr at 37°C and alkylation with 20mM [^{14}C] iodoacetamide (50μCi) for 2hr at 0°C.

SDS/PAGE under reducing conditions was as described by Laemmli [11] using a 15% polyacrylamide slab gel; molecular weight markers were ovalbumin (43,000), glyceraldehyde 3-phosphate dehydrogenase (37,000), immunoglobulin L chain (25,000), cytochrome C (12,000), bovine trypsin inhibitor (6,000), and bovine insulin (3,000). Samples for amino acid analysis (1μg) were hydrolysed in 6M HCl for 24hr at 110°C and were analysed using a Beckman 6300 amino acid analyzer. N-terminal amino acid sequences were determined using an Applied Biosystems 470A sequenator; the PTH-amino acids were identified and quantitated by reverse phase HPLC on a Dupont Zorbax C_8 column [12] or by using an Applied Biosystems 120A analyzer. Computer searches for amino acid sequence homologies were carried out on the May 1985 National Biomedical Research Foundation data base, using the global search program of Wilbur & Lipman [13].

RESULTS

The EGTA extracts of pig intestinal epitheliam and mesenteric lymph node lymphocyte plasma membranes were fractionated on a mono S column. The polypeptide compositions of the fractions eluted with 0.26M NaCl are

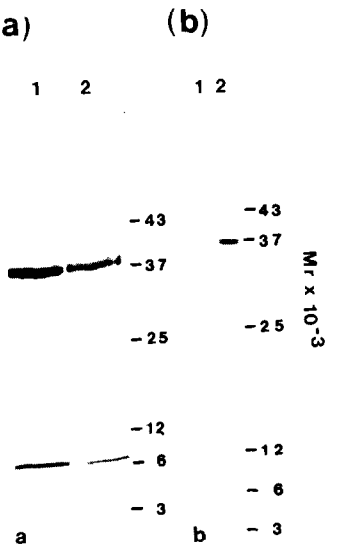

Fig.1 Polypeptide compositions revealed by SDS/PAGE (a) Fractions eluted with 0.26M NaCl by Mono S column chromatography of EGTA extracts of pig mesenteric lymph node lymphocyte plasma membrane (track 1) and intestinal epithelium brush border membrane (track 2). (b) purified 10,000-Mr (track 1) and p36 (track 2) polypeptides isolated by gel filtration. Polypeptides were detected with Coomassie blue. The positions of the molecular weight markers are indicated.

illustrated in Fig.1a. In each case, SDS/PAGE revealed two polypeptides only of about 36,000 and 6,000-Mr. The higher Mr polypeptides were identified as p36 by Western blotting with a rabbit anti-(chicken fibroblast p36) serum (results not shown). Gel filtration on a column of Superose 12 in 0.15M NaCl, 10mM Na phosphate buffer pH 7.3, 2mM EGTA, 1mM DTT, showed that each fraction comprised principally one species with an apparent Mr of about 70,000. Some dissociation into the constituent polypeptides was also detected (results not shown).

The 36,000 and 10,000-Mr polypeptides were separated either by preparative SDS/PAGE or, after reduction and alkylation, by gel filtration in 6M guanidine HCl (Fig.1b). The gel filtration pattern of the pig mesenteric lymph node lymphocyte fraction revealed two peaks whose positions corresponded to molecular weights of about 36,000 and 9,000, as calculated according to Fish et al. [14] relative to the elution positions of standard proteins run under identical conditions. Amino acid analyses of the material

Table 1. Amino acid compositions of p36 and the 10,000-Mr polypeptides

	Polypeptide					
	p36			10,000-Mr		
	L	E	*	L	E	*
Cys	1.0	1.0	1.6	1.2	1.3	2.2
Asx	10.4	11.0	11.1	9.1	9.8	10.5
Thr	4.9	5.1	5.1	4.0	3.4	4.0
Ser	7.1	8.8	8.9	5.1	3.0	3.2
Glx	11.7	12.3	12.3	12.2	13.2	12.8
Pro	1.9	2.2	2.9	3.9	3.5	3.7
Gly	9.2	8.4	6.2	14.1	9.2	6.6
Ala	6.8	7.0	7.0	6.9	6.0	5.9
Val	5.3	5.1	4.5	6.0	4.8	3.9
Met	2.2	2.1	2.9	4.2	6.8	7.1
Ile	5.4	5.5	4.6	3.2	3.2	2.9
Leu	9.6	9.2	9.2	7.7	9.2	7.9
Tyr	4.8	4.4	4.6	1.8	1.0	2.2
Phe	2.3	2.2	3.4	7.1	9.2	9.1
His	1.5	1.5	1.1	2.4	3.2	3.1
Lys	8.5	8.1	9.5	8.8	12.7	12.8
Arg	5.5	5.1	5.1	3.4	2.4	2.3

Values are expressed as mol % and represent the average of duplicate estimations. L, lymphocyte; E, epithelial; * indicates values determined previously [7] for pig intestinal epithelium. Cys was determined as S-carboxymethylcysteine.

recovered from the above peaks indicated the presence of equimolar amounts of the two polypeptides.

Table 1 shows the amino acid compositions of the 36,000 and 10,000-Mr polypeptides purified by gel filtration. The values for the intestinal epithelium polypeptides agree, essentially, with those published previously [7], and the values for the lymphocyte polypeptides do not differ significantly from those of the epithelial polypeptides. The most striking differences between the amino acid compositions of the 36,000 and 10,000-Mr polypeptides are the higher proline, methionine and phenylalanine, and the lower tyrosine contents of the smaller polypeptide.

Polypeptides purified by gel filtration and SDS/PAGE were used for N-terminal sequence analysis. No meaningful signals were obtained for 100pmol of the 36,000-Mr polypeptide purified from lymphocytes and epithelium by gel filtration, suggesting that this polypeptide has a blocked N-terminus. Figure 2 shows the results obtained for 2nmol of the lymphocyte 10,000-Mr polypeptide purified by gel filtration; the average yield of PTH-amino acid for the first 10 cycles was about 10% of the protein loaded onto the sequenator. An identical but longer sequence was obtained using 2nmol of the

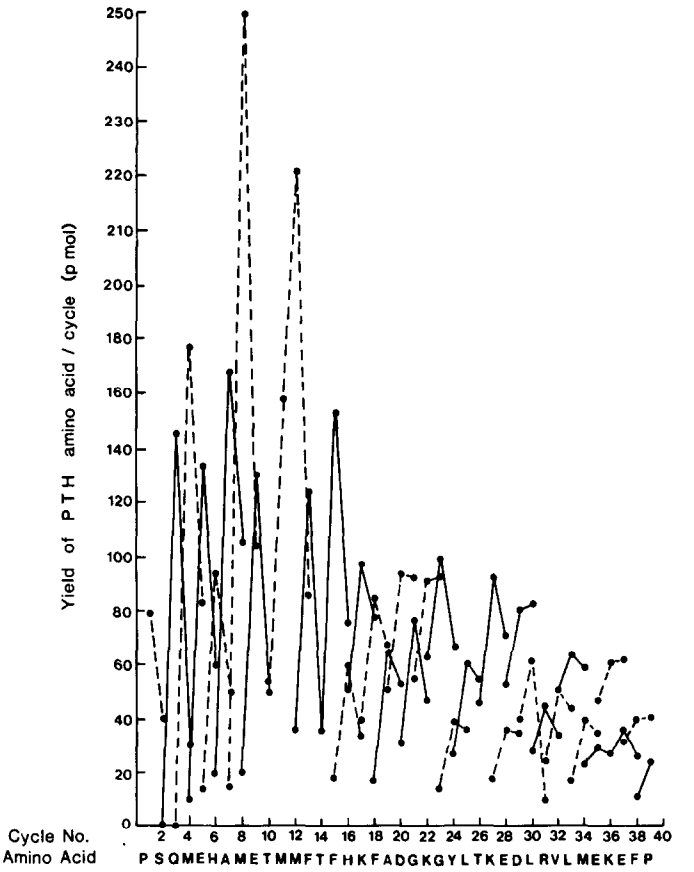

Fig.2 N-terminal amino acid sequence of the lymphocyte 10,000-Mr polypeptide expressed as yield of PTH-amino acid per cycle.

epithelium 10,000-Mr polypeptide (Fig.3). The latter results were in part (residue no. 13 to 30 and 35 to 51) confirmed by sequence analysis of fragments obtained by cleavage with CNBr (results not shown). Polypeptide purified by SDS/PAGE proved much less suitable for sequence determination than the gel filtered material. Thus, 3nmol of the epithelium polypeptide eluted from SDS/PAGE gave <1% recovery of PTH-amino acid relative to the amount of polypeptide added, and the sequence did not extend beyond residue number 12. These results indicate that the SDS/PAGE purified polypeptide is much less suitable for N-terminal sequence determination, because of blockage of the N-terminus probably due to reactive groups in the polyacrylamide gel.

As shown in Fig.3, a computer search of the protein amino acid sequence data base revealed homologies of 56 and 42% with the N-terminii of the α and

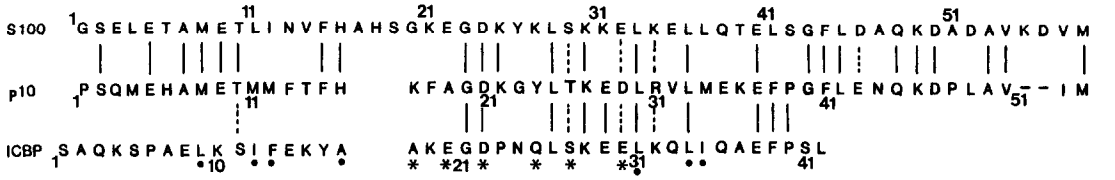

Fig.3 Comparison of the N-terminal amino acid sequence of the 10,000-Mr polypeptide with those of the α chain of bovine S100 [14] and pig intestinal Ca^{2+}-binding protein (ICBP) [15]. The sequence of the 10,000-Mr polypeptide has been displaced at residue no.16 three places to the right in order to maximise the subsequent homology with S100. No positive identification of the residue was made at positions no.52 and 53. The sequence of ICBP is not extended beyond residue no.42, because the sequence separating the EF hands is shorter than in S100. ——, identical residues; ---, conserved residues; *, residues involved in Ca^{2+}-binding; •, hydrophobic, α-helical residues.

β chains respectively of bovine S100 protein [15] and a 38% homology with the N-terminal sequence of pig intestinal Ca^{2+}-binding protein [16].

DISCUSSION

The lymphocyte p36 polypeptide has been shown previously to be preferentially associated with the nonionic detergent insoluble fraction of the plasma membrane and to be solubilised by Ca^{2+}-chelators [5]. The present work indicates that it interacts with a smaller polypeptide of Mr 6,000 or 9,000, as judged by SDS/PAGE and gel filtration in guanidine HCl respectively (Fig.1). p36 isolated from pig intestinal epithelium bound Ca^{2+}, in the presence and absence of the lower Mr polypeptide [17]. The separated lower Mr polypeptide did not, however, apparently bind Ca^{2+}, as judged by fluorescence spectroscopy.

The 10,000-Mr polypeptide isolated from pig lymphocytes and intestinal epithelium possessed identical N-terminal amino acid sequences, which showed homology with the N-terminus of the single polypeptide comprising ICBP[16] and the α and β subunits of S100, which also binds Ca^{2+} [15] (Fig.3). Indeed, it appears on the basis of this homology that the 10,000-Mr polypeptide should bind Ca^{2+}, in contrast to the above result [17]. Thus, residues no. 8-37 should, by analogy with residues no.11-40 of S100 α chain and 9-38 of ICBP, form a "pseudo EF hand", which in ICPB has been shown to bind Ca^{2+} with high affinity [18,19]. Furthermore, residues no. 51, 54 and 55 appear to form the beginning of the counterpart of the "EF hand", which is located in the C-terminal domain of S100 and ICPB [19].

S100 protein is present mainly in glial cells, but has also been identified by immunohistology in several non-neural elements including Langerhans and lymphatic recticular cells, and a sub-population of human T8+ lymphocytes [20]. It is questionable whether the extent of amino acid sequence homology with the α and β subunits (56 and 42% respectively), revealed in the present studies, would result in immunological cross-reactivity, but it appears conceivable that some of the claims for the presence of S-100 protein in non-neural cells may actually represent the 10,000-Mr polypeptide. S100 protein appears to play a regulatory role in microtubule assembly and disassembly [21]. A related role for the 10,000-Mr polypeptide and its associated p36 polypeptide in respect of the cell cytoskeleton is suggested by their preferential association with the nonionic detergent insoluble fraction of the cell surface membrane.

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