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Molecular Analysis of Pleckstrin: The Major Protein Kinase C Substrate of Platelets

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Activation of protein kinase C (PKC) in platelets causes the immediate phosphorylation of pleckstrin, an apparent M_r 40–47,000 protein previously called 40K or P47. Pleckstrin presumably plays an important but as yet unknown role in mediating cellular responses evoked by agonist-induced phosphoinositide turnover. We have cloned the cDNA for pleckstrin from the HL-60 human promyelocytic leukemia cell line by immunological screening of a λgt11 expression library (Tyers et al.: *Nature* 333:470–473, 1988) and now report further analysis of the pleckstrin sequence. Pleckstrin has a deduced M_r of 40,087 and is encoded by a 1,050-bp open reading frame which is preceded by a short open reading frame that terminates before the correct initiator methionine. A single polymorphic site was found in the coding region. An unusual pattern of sequence heterogeneity occurred about a poly(A) tract in the 3′ untranslated region. The 3.0-kb pleckstrin mRNA induced upon differentiation of HL-60 cells apparently has heterogeneous 5′ ends which undergo differential regulation during HL-60 cell maturation. Analysis by multiple sequence alignment with known PKC substrates identified a strong candidate site for phosphorylation by PKC and a potential Ca^{2+}-binding EF-hand motif. No other similarities to proteins in current databases were found.

Key words: calcium-binding, cDNA sequence, PKC substrate, phosphorylation, P47

Signal transduction mediated by hydrolysis of phosphatidylinositol 4,5-bisphosphate involves the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which activate protein kinase C (PKC) and release Ca^{2+} from intracellular stores, respectively [1]. In platelets, an early event in response to agonists such as thrombin which initiate phosphoinositide turnover is the marked phosphorylation by PKC of a protein of apparent M_r 40,000–47,000 previously called 40K or P47 [1–3]. Based on its specific expression in platelets and various differentiated white blood cells, we proposed the name pleckstrin for this protein, for platelet and leukocyte C kinase substrate, and for KFARKSTRRSIR, the most probable phosphorylation site [4].

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The role of pleckstrin in stimulus-response coupling is not known, although its phosphorylation usually correlates with subsequent secretion of platelet granule contents [3]. Based on its immunoreactivity with antirenocortin antibody and partial inhibition of phospholipase A_2 activity by platelet fractions containing pleckstrin, it was postulated that pleckstrin is a member of the lipocortin family [5]. In addition, the copurification of pleckstrin with inositol trisphosphate 5-phosphomonoesterase activity from platelets led to the suggestion that phosphorylated pleckstrin attenuates Ca^{2+} release from intracellular stores by degrading inositol 1,4,5-trisphosphate [6]. It has also been suggested that pleckstrin is the α-subunit of the mitochondrial protein pyruvate dehydrogenase [7]. Finally, it has been claimed that partially purified pleckstrin inhibits actin polymerization in its nonphosphorylated form, implicating it in control of cytoskeletal reorganization during secretion [8]. To help elucidate pleckstrin structure and function, we have cloned pleckstrin cDNA from HL-60 cells [4], a human promyelocytic leukemia cell line [reviewed in 9] that becomes enriched for pleckstrin when induced to differentiate with retinoic acid [10]. The deduced amino acid sequence of pleckstrin shows that it is a unique protein and clearly unrelated to lipocortins or pyruvate dehydrogenase. We report here details of further sequence analysis and of the expression of pleckstrin and its cDNA.

MATERIALS AND METHODS

Cell Culture

The HL-60 human promyelocytic leukemia cell line was obtained from the American Type Culture Collection and grown as described previously [11]. All experiments were carried out with cells between passage numbers 20 and 50. Differentiation was accomplished as described previously [10,11] by the addition of drugs in minimal volumes of carrier solvent. Differentiation was assessed visually and by cell growth and enzymatic markers [10,11].

Construction and Screening of cDNA Libraries

The original library in which pleckstrin clones were identified was constructed from poly $(A)^+$ RNA isolated from HL-60 cells treated with retinoic acid for 5 days. The cDNA for this library was made using a modification of the classical S1 method in order to optimize the chance of obtaining fusion proteins which would lead to immunological reactivity with rabbit pleckstrin antiserum [4]. Three clones discussed in this report (clones 34A, 34B, and 37) originated from the initial library. In an attempt to obtain longer 5′ pleckstrin cDNA clones, two more libraries were constructed in a similar manner, but instead cDNA was made according to Gubler and Hoffman [12] to favor production of full-length clones. λgt11 (Promega Biotech, Madison, WI) was used as the vector for one library, while the other was made in λZAP (Stratagene, San Diego, CA). Both were screened with previous pleckstrin clones under the hybridization conditions outlined below for Northern analysis. We report here analysis of one clone from the λgt11 library (clone 3-1) and three from the λZAP library (clones 2A1, 2A3, and 3A1). Positive λgt11 recombinants were subcloned as a Kpn I-Sac I fragment (i.e., a complete cDNA insert flanked by λgt11 sequences) into pUC 118/119 and sequenced by dideoxy chain termination (Sequenase, USB, Cleveland, OH) using specific primers [13] and/or nested sets of deletions

[14]. λZAP clones were excised as described by the supplier to create Bluescript plasmids containing pleckstrin inserts. In this case partial sequencing was carried out from either primers complementary to known pleckstrin sequences or from the M13 reverse primer.

Production of Recombinant Pleckstrin in E. coli

The Pvu II-Nco I fragment of pleckstrin cDNA (nucleotides −53 to 1091) was inserted into the bacterial expression vector pKK-233 [15] using Nco I linkers to give a construct expressing the complete pleckstrin open reading frame preceded by the 5′ extension. Including two amino acids contributed by linker sequence, this corresponded to an M_r of 42,112. In a second construct, the G at −2 with respect to the putative initiator ATG (nucleotide −2) was mutated [16] using a mismatch oligonucleotide 5′-GTTCCATGGTGGCTGGA-3′. Mutant clones were identified by colony hybridization to end-labelled mutant oligonucleotide in the presence of tenfold excess cold wild-type oligonucleotide that was fortuitously made in the same region for use as a sequencing primer. The mutated sequence contained a new Nco I site at the first methionine which allowed direct cloning of the pleckstrin coding region into the translation initiation site of pKK-233. *Escherichia coli* JM109 containing each construct were grown to saturation overnight. Cells were pelleted, lysed in sample buffer, electrophoresed on an 11% polyacrylamide-SDS gel, and stained with Coomassie blue. Authentic size pleckstrin accumulated up to 20% of bacterial protein, whereas N-terminally extended pleckstrin reached at most 1%. Considerable variability in the level of recombinant protein was observed, as expression declined dramatically upon restreaking of transformed cells or upon prolonged storage of cells on agar plates. Uniformly high expression was only achieved with freshly transformed cells.

Northern Analysis

Total RNA was isolated using guanidine thiocyanate as described previously [17], electrophoresed (15 μg) on a 1% agarose, 6% formaldehyde gel, transfered to nitrocellulose, and probed with either 10^7 cpm of random primed [18] pleckstrin cDNA Pvu II fragment (nucleotide −53 to 2024) or an oligonucleotide complementary to a conserved region of actin mRNA. Hybridization with cDNA probes was in 50% formamide, 5× Denhardt's solution, 5× SSC, 0.1% SDS, 50 mM NaH_2PO_4, 5 mM $Na_2H_2P_2O$, and 250 μg/ml salmon sperm DNA at 48°C. Blots were washed with 2× SSC, 0.1% SDS at room temperature, then 0.1× SSC, 0.1% SDS at 48°C. The oligonucleotide probe was used as described elsewhere [17].

Primer Extension Analysis

Reverse transcription of 1 μg of poly $(A)^+$ RNA was carried out under the conditions described by Wickens et al. [19] except that 0.15 pmol of primer complementary to nucleotides 155–170 was substituted for oligo (dT). The primer was end-labeled to a specific activity of 3×10^6 dpm/pmol with T4 polynucleotide kinase and annealed to the RNA under reaction conditions for 10 min prior to addition of 20 U of reverse transcriptase. Reactions were terminated with an equal volume of dye loading buffer and electrophoresed on a 5% acrylamide, 8 M urea denaturing gel using an end-labeled 1-kb ladder (BRL, Gaithersburg, MD) as a marker. The gel was dried and exposed to XAR film overnight at −70°C with an intensifying screen.

Sequence Analysis

Database searches and sequence analyses were carried out using the NBRF Protein Identification Resource (Georgetown, MD) and the University of Wisconsin GCG software (processed on the National Research Council CND/SND system, Ottawa, Ontario). For multiple sequence alignment, we used modifications of the Bacon and Anderson [20] ALIGN program on a VAX 8600 (Harley et al., in preparation). In brief, to search for potential PKC phosphorylation sites, the similarity matrix [20] was modified to favor Ser/Thr and Arg/Lys matches. The ALIGN program was then run, four sequences at a time, on the eight known substrates of PKC listed in Figure 8a, and the highest scoring subset was chosen as the reference set. The other sequences, including the top scoring regions of pleckstrin, are aligned against the reference set (Fig. 8a), and shown with their scores and the normalized score of the core set. To search for potential Ca^{2+}-binding EF-hand motifs the ALIGN program was run with a window size of 30 residues and only residues in the conserved positions described by Kretsinger [21] contributed to the overall score. Furthermore, residue scores corresponding to the loop region were weighted twice as heavily as scores in the flanking helix regions. The core set against which the sequences in Figure 8b were individually aligned consisted of the four proteins for which crystallographic data unambiguously assigns residues to the EF hand: calmodulin [22], intestinal calcium-binding protein [23], troponin C [24], and parvalbumin [25].

RESULTS AND DISCUSSION

Pleckstrin cDNA clones were obtained by immunological screening of a λgt11 library constructed from mRNA from HL-60 cells differentiated with retinoic acid for 5 days [4]. Several cDNA inserts (spanning 2.75 kbp) were sequenced and all were consistent with peptide sequence obtained from purified human platelet pleckstrin, as we reported previously [4]. The first ATG of our original clones initiated a long open reading frame encoding a 350 amino acid protein that comigrated with authentic pleckstrin when expressed in vitro and in *E. coli* [4]. Sequencing of subsequent clones has now extended the 5′ end of the cDNA by 25 nucleotides revealing a short open reading frame of 21 nucleotides beginning upstream from, but out of frame with, the pleckstrin initiator ATG (Fig. 1). Although clearly not part of the pleckstrin sequence, the context of this potential translation start is similar to that of known initiator sequences [26] and could play a role in regulation of pleckstrin expression. Such upstream ATG signals are relatively rare in vertebrate mRNAs (<10%), but occur frequently (60–70%) in mRNAs encoding oncogenes [26]. To account for translation initiation after an upstream ATG, Kozak [27] has proposed a re-initiation model in which the length of the intercistronic region dictates the efficiency of translation at the downstream ATG. For preproinsulin, a minimum of 79 nucleotides were required between the termination codon of the short upstream open reading frame and the authentic ATG to promote efficient translation [27]. The intercistronic region for pleckstrin is only 46 nucleotides, shorter than might be anticipated. In HL-60 cells, one upstream ATG is found in the myeloperoxidase mRNA [28], while four are found in the α-tubulin message [29]. In contrast to pleckstrin mRNA which increases upon differentiation (see below and ref. 4), both of these mRNAs are down-regulated dur-

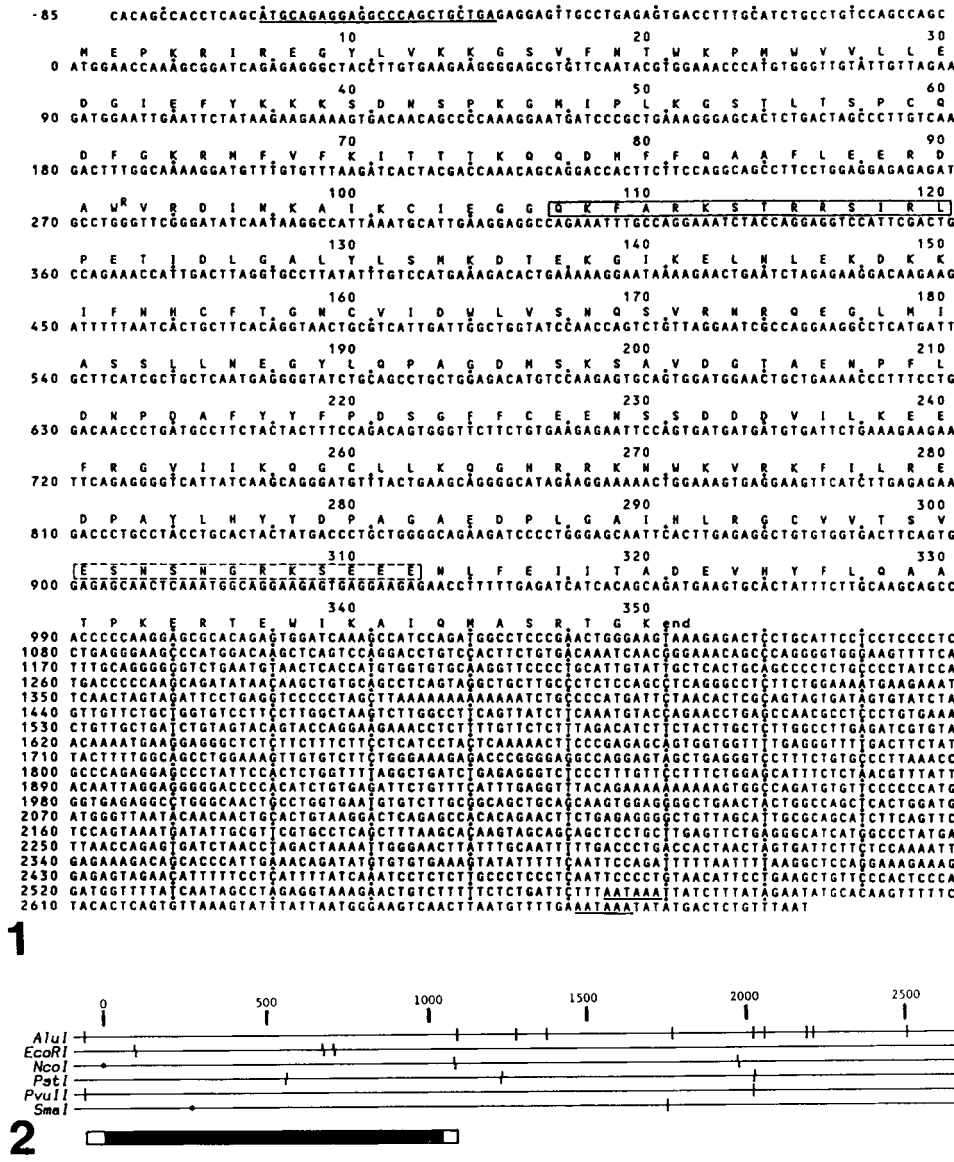


Fig. 1. Nucleotide and deduced amino acid sequence of pleckstrin cDNA. The 350 amino acids of pleckstrin encoded by the 1,050-nucleotide open reading frame are shown in capital one-letter code. The nucleic acid sequence is numbered relative to the first base of the coding region, which is assigned coordinate 0. The short open reading frame 5′ to the start of the pleckstrin coding sequence is underlined, as are possible polyadenylation signals at the 3′ end of the sequence. The best candidate PKC phosphorylation site (closed box) and the putative EF-hand Ca^{2+}-binding loop (open box) are indicated. The single base change at nucleotide 273 resulting in a W to R substitution observed in two clones (2A1 and 3A1) is shown by superscript R at residue number 92.

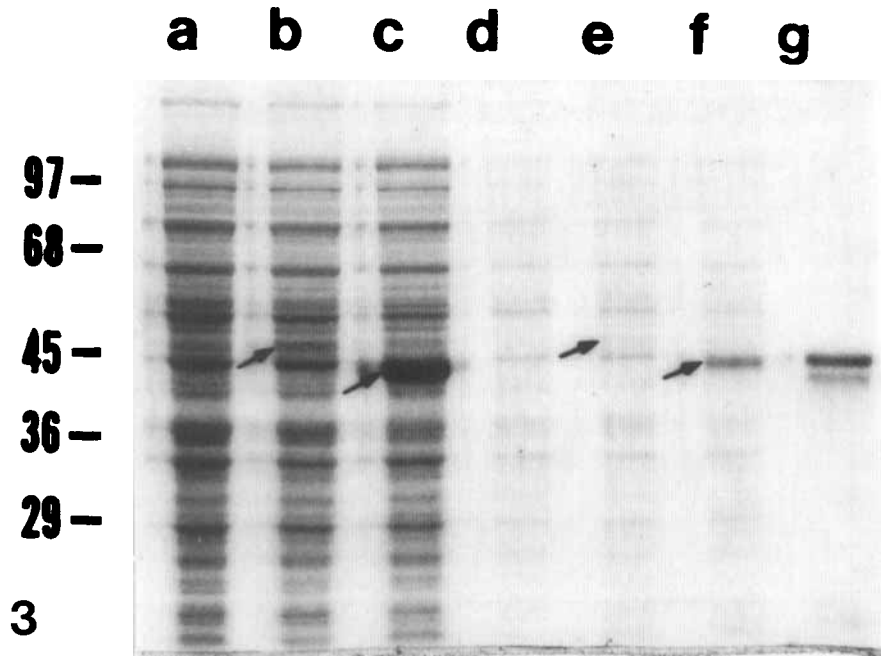
Fig. 2. Restriction map of pleckstrin cDNA showing sites used in cloning and subsequent manipulations. The Nco I site created by site directed mutagenesis in order to clone the complete coding region, and the polymorphic Sma I site are indicated by *. The bar below represents cDNA fragments (Pvu II-Nco I, or Nco I*-Nco I) expressed in *E. coli;* the solid area is the pleckstrin coding region.

ing HL-60 differentiation, although α-tubulin expression actually increases due to superimposed translational control [29].

Initiation of pleckstrin translation as indicated in Figure 1 is based on in vitro transcription and translation of pleckstrin cDNA and expression of the pleckstrin coding region from the bacterial expression vector pKK-233 [4]. In addition, the context of the ATG at nucleotides 0-2 is most like the consensus sequence for initiation of translation (CCAGCATGG versus CCACCATGG). Pleckstrin cDNA fragments expressed in *E. coli* are shown below the cDNA restriction map (Fig. 2). Analysis of products showed that recombinant pleckstrin (Fig. 3c,f, arrows) comigrates with platelet pleckstrin (Fig. 3g, upper band), whereas a separate construct encoding pleckstrin as well as the next 20 N-terminal amino acids of the open reading frame gave a product 2.6 kDa larger than pleckstrin (Fig. 3b,e, arrows). In this gel system, platelet pleckstrin migrates with an apparent M_r of 44,000. The lower band seen in Fig. 3g represents P42, a pleckstrin-related protein that copurifies with platelet pleckstrin. Slight differences in apparent M_r between recombinant pleckstrin and platelet pleckstrin standard may arise from band compression in the overloaded gels used for Coomassie staining or from post-translational modification. Thus, the deduced M_r of pleckstrin is 40,087. Interestingly, high-level expression was only obtained with the correct pleckstrin coding sequence, not the 5′-extended construct. In neither case was expression affected by the presence of IPTG, an inducer of the lac promoter (not shown). This may reflect insufficient lac repressor in bacteria transformed with high-copy-number plasmids.

The sequence of independent pleckstrin cDNA clones has revealed heterogeneity in both the coding and 3′ noncoding regions. A single base difference at nucleotide 273 caused a tryptophan to arginine substitution in two of five independent clones sequenced (Fig. 1). The different clones encode proteins with predicted pI values that differ by 0.3 pH units, close to observed differences for pleckstrin isoforms seen on two-dimensional gels [30]. Clone heterogeneity may represent natural human polymorphism or a mutation accumulated in the HL-60 cell line. The former explanation is likely correct since restriction fragment length polymorphism has been detected in the human population with MspI, an enzyme that cuts within the new SmaI site (Fig. 2) created by this base substitution (data not shown). Heterogeneity observed in the long 3′ untranslated region of pleckstrin mRNA was confined to bases near one of two poly(A) tracts (nucleotides 1384–1396 and 1947–1956). The first of these was surrounded by a pattern of single base differences amongst different clones (Fig. 4). Since the clones were from three independent libraries and the differences were clearly not random (i.e., each clone appears to have a subset of the same mutations), it is unlikely that the heterogeneity reflects a cloning artifact. It is also unlikely that the HL-60 line is polyploid for the pleckstrin locus since it is not grossly rearranged or amplified compared to normal human leukocyte DNA and since Southern analysis suggests it is a single copy gene [4]. This variation may have been generated during transcription or processing of pleckstrin mRNA.

All positive recombinants from immunological screening and nucleic acid hybridization contained inserts which hybridized to a 3.0-kb mRNA (Fig. 6). This transcript was up-regulated to varying levels during both granulocytic HL-60 differentiation induced by retinoic acid [31] dibutyryl cAMP [32], and monocytic differentiation induced by 12-O-tetradecanoyl phorbol acetate (TPA) [33], TPA in synergistic combination with A23187 [11], or 1,25-dihydroxyvitamin D_3 [34]. Primer exten-



Clone Sequence

34A TGAGGTCCCCCTAGCTTAAAAAAAAAAAAATCTGCCCCATGATTCT
34B TGAGGTCTCCCCAGCTT•AAAAAAAAAAAAATCTGCCCCGTGATTCT
37 TGAGGTCTCCCCAGCTTAAAAAAAAAAAAATCTGCCCCGTGATTCT
3-1 TGAGGTCTCCCCAGCTTAAAAAAAAAAAAATCTGCCCCGTGATTCT

3A1 TGAGGTCCCCCTAGCTTAAAA

4 2A3 AAAAAAAAAAAAATCTGCCCCATGATTCT

Fig. 3. Expression of the pleckstrin coding region in *E. coli*. Pleckstrin open reading frames were inserted in the pKK-233 prokaryotic expression vector [15] and lysates representing protein from 8 μl (**a–c**) or 0.8 μl (**d–f**) of *E. coli* grown to saturation were analysed by SDS-PAGE and Coomassie blue staining. Lysates were from cells transformed with plasmid pKK-233 containing **a,d)** no insert; **b,e)** pleckstrin cDNA nucleotides −53 to 1091 corresponding to M_r 42,112; **c,f)** pleckstrin cDNA nucleotides 0 to 1091 corresponding to M_r 40,087. Platelet pleckstrin (0.5 μg) is shown in lane **g.** Protein marker sizes (kDa) are indicated on the left.

Fig. 4. Alignment of independent pleckstrin clones about a 3′ untranslated poly (A) tract (nucleotides 1383–1395) to show multiple single base changes in each clone. Partial clones 3A1 and 2A3 apparently initiated and terminated cDNA synthesis, respectively, at this poly (A) tract. Differences from clone 34A are shown by a cross. The dot represents a deleted base.

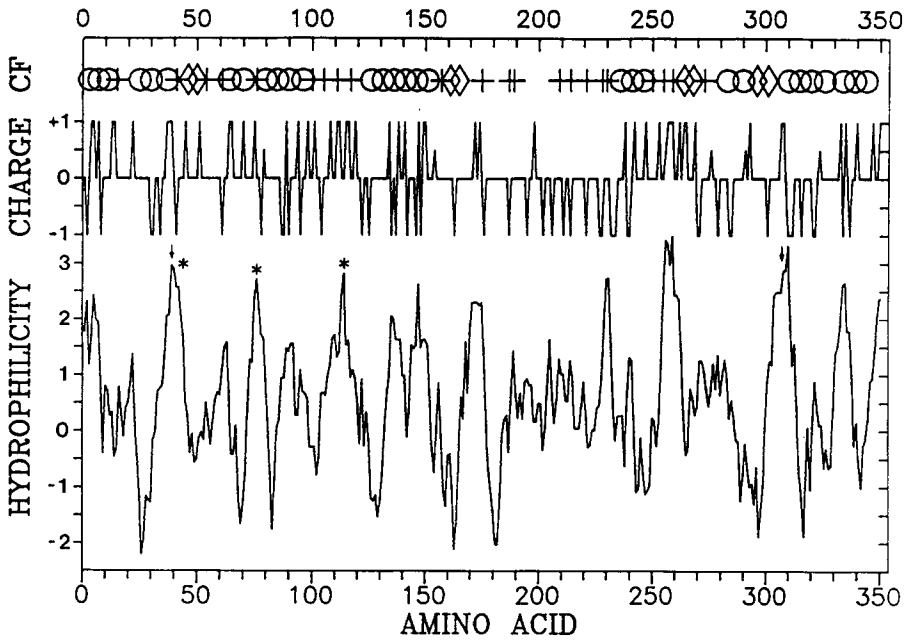


Fig. 5. Predicted secondary structure, charge profile, and hydrophilicity plot of pleckstrin protein. Secondary structure and hydrophilicity were determined with standard parameters on the University of Wisconsin Genetic Computer Group software using the methods of Garnier et al. [46] and Kyte and Doolittle [47], respectively. Secondary structure symbols represent α-helix (o), β-sheet ($\diamond$), and turn (+). The charge profile was plotted by assigning +1 to R or K residues, +0.5 to H, and −1 to E or D residues. Potential proteolytic sites that could yield P42 (see text) are indicated by arrows. Probable PKC phosphorylation sites are indicated by asterisks.

sion analysis from an oligonucleotide 256 bases downstream of the 5′ end suggested that the 3.0-kb transcript was actually composed of 4 predominant forms which differ slightly in the length of the 5′ end (Fig. 7). These transcripts were differentially regulated during HL-60 cell differentiation. The two intermediate size transcripts (corresponding approximately to presumptive cap sites at nucleotide −100 and −120) decline in relative abundance such that the shortest and longest transcripts (capped at nucleotide −70 and −170) predominate in differentiated cells. If these differences are not artifacts of the primer extension analysis, they may represent differential splicing or alternate cap sites in pleckstrin mRNA which could play a regulatory role in translation [see refs. 35,36]. Although sequence data for the promoter region is not available, southern analysis with the most 5′ Eco RI fragment of pleckstrin cDNA indicates that this exon region is spaced over 14 kbp of the human genome [ref. 4] (unpublished) and may thus derive from complex mRNA processing events.

Primer extensions were originally carried out to determine how much 5′ region was missing from our cDNA clones. The largest clone thus includes the presumptive shortest transcript but may be up to 85 nucleotides short of the longest pleckstrin mRNA. The upstream ATG may not be functional in the short transcript since it nearly corresponds to the presumptive cap site. It thus might be under different translational control than the longer transcripts, perhaps increasing expression of pleckstrin

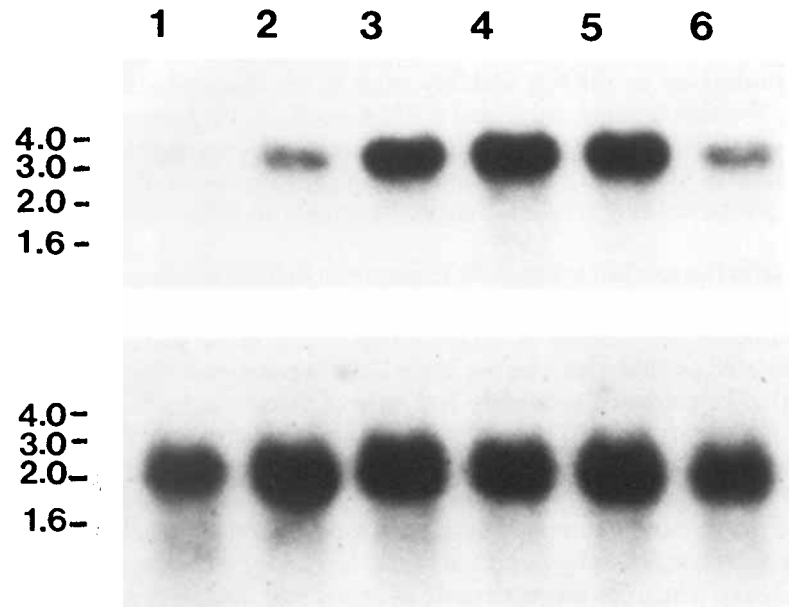


Fig. 6. Regulation of pleckstrin mRNA during HL-60 differentiation. Total RNA from either untreated cells (**lane 1**) or cells treated with either 20 nM TPA for 2 days (**lane 2**), 1 nM TPA and 400 nM A23187 for 2 days (**lane 3**), 1 μM retinoic acid for 7 d (**lane 4**), 100 nM 1,25-dihydroxyvitamin D_3 for 7 days (**lane 5**), or 500 μM dibutyryl cAMP for 3 days (**lane 6**) was probed with the pleckstrin Pvu II cDNA fragment (**top**) or an oligonucleotide complementary to a conserved region of α-, β- and τ-actin mRNA as a normalization control (**bottom**). Marker sizes (nucleotides) are indicated on the left.

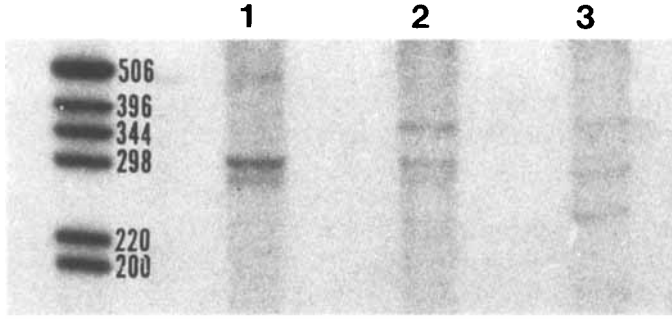


Fig. 7. Analysis of the 5′ end of pleckstrin mRNA and regulation during HL-60 differentiation. Poly(A)$^+$ RNA from undifferentiated cells (**lane 1**), cells treated with 20 nM TPA for 16 h (**lane 2**), or cells treated with 1 μM retinoic acid for 5 days (**lane 3**) was reverse-transcribed from a ^{32}P-end-labeled primer. The primer used begins 256 nucleotides from the 5′ end of the cDNA. The predominant bands thus correspond approximately to nucleotide positions −70, −100, −120, and −170. Marker sizes (nucleotides) are indicated on the left.

upon differentiation in a manner analogous to the HL-60 α-tubulin system [27]. However, pleckstrin appears to be regulated during HL-60 differentiation primarily at the level of transcription or mRNA stability since in all cases protein abundance, as detected by Western blotting, paralleled mRNA levels [4,10]. Increased expression of pleckstrin as HL-60 cells differentiate into mature white cells correlates with the tissue distribution of pleckstrin mRNA and protein [4] (Stewart et al., in preparation), suggesting pleckstrin may confer a function common to differentiated hemopoietic cells.

Pleckstrin has marked hydrophilic regions rich in basic residues at both N and C termini (Fig. 5, arrows) which could be sensitive sites for proteolysis. If pleckstrin were cleaved at either of these sites, it would generate a species of the correct size to account for P42, a related protein with which it copurifies (Stewart et al., in preparation; also see Fig. 3g). The predicted secondary structure of pleckstrin indicates it is 37% α-helical and is highly polar (103 of the 350 amino acids are charged, Fig. 5), consistent with its cytosolic location in platelets and HL-60 cells (not shown).

Since pleckstrin is heavily phosphorylated by PKC in stimulated platelets [1–3], we searched the deduced amino acid sequence for potential phosphorylation sites. It is known that phosphorylation by protein kinase C has a strong requirement for positive residues [37–41]. Thus, the preponderance of serine and threonine residues in pleckstrin adjacent to basic residues may account for its high level of phosphorylation [30]. Although multiple, phosphorylated forms of pleckstrin from stimulated platelets are resolved by 2-D gel electrophoresis [30], the exact number of phosphorylation sites is not known. Multiple sequence alignment [20] against known high-affinity peptide substrates of PKC picked residues 107–125 of pleckstrin as the most likely region of phosphorylation (Fig. 1, box, 8a). This sequence is very similar to the PKC pseudosubstrate site [38], the serine analogue of which is potently phosphorylated by PKC. Several alignments of residues 107–125 are possible, all of which score as well as or better than the known high-affinity substrate sites (Fig. 8a). Thus, this region may be phosphorylated at multiple sites (Ser^{113}, Thr^{114}, or Ser^{117}), perhaps analogous to the phosphorylated region of myelin basic protein [39] and smooth muscle myosin light chain [41]. Lower-scoring potential phosphorylation sites in pleckstrin are also listed in Figure 8a.

Sequence analysis also revealed a potential Ca^{2+} binding EF-hand motif [21] at the C-terminus of pleckstrin (Fig. 1, dashed box, 8b). The putative pleckstrin EF-hand is conserved at all Ca^{2+} liganding residues but has an anomalous lysine in place of a hydrophobic residue in the middle of the Ca^{2+} binding loop (Fig. 8b). According to the criteria of Kretsinger [21], the pleckstrin EF-hand may only marginally bind Ca^{2+} even though it scores higher than that predicted for fibrinogen, which is known to bind Ca^{2+} [42]. Proteins with similar scores (p11 [43] and chicken α-actinin [44]) which appear not to bind Ca^{2+} have lost at least one essential oxygen liganding residue, whereas pleckstrin has not (Fig. 8b). If anomalous amino acids are permitted within the Ca^{2+} binding loop, as suggested by other alignments of fibrinogen [42] and the rat vitamin-D-dependent calcium-binding protein [45] by "looping-out" the contrary residue, then the pleckstrin sequence becomes an even stronger candidate for an EF-hand by discounting lys^{308} and shifting over the C-terminal residues. The pleckstrin sequence yields no further direct clues as to other possible functions; searches for weak consensus sequences such as actin or nucleotide binding domains were unsuccessful.

(a)

		SCORE	Ref.
IL-2-R (241-250)	Q R R Q R K S R R T	450	37
SM-MLC (3-12)	K R A K A K T T K K	450	41
PKCPS (19-28)	R F A R K G S L R Q	450	38
RS6 (230-239)	K R R R L S S L R A	450	40
EGFR (648-657)	H I V R K R T L R R	441	37
P60 (5-14)	S K P K D P S Q R R	431	37
MBP (104-113)	G K G R G L S L S R	408	39
P36 (19-28)	P P S A Y G S V K A	392	37
PLE (108-117)	K F A R K S T R R S	470	
(107-116)	Q K F A R K S T R R	455	
(111-120)	R K S T R R S I R L	450	
(37-46)	K K K S D N S P K G	433	
(34-43)	E F Y K K K S D N S	427	
(67-76)	F V F K I T T T K Q	416	

(b)

i)	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	SCORE		Ref.
ii)	E	h			h	h			h	d		d		d	G		i	d			E	h			h	h			h	KI		AIN
iii)											X		Y		Z		-Y		-X		-Z											
PLE	L	R	G	C	V	V	T	S	V	E	S	N	S	N	G	R	K	S	E	E	E	N	L	F	E	I	I	T	A	11	1607	
CaM	E	F	K	E	A	F	S	L	F	D	K	D	G	N	G	T	I	T	T	L	E	L	G	T	V	M	R	S	L	16	1781	22
S100	V	V	D	K	V	M	E	T	L	D	S	D	G	D	G	E	C	D	F	Q	E	F	M	A	F	V	A	M	I	14	1720	43
p11	A	V	D	K	I	M	K	D	L	D	Q	C	R	D	G	K	V	G	F	Q	S	F	F	S	L	I	A	G	L	12	1685	43
αACT	E	F	A	R	I	M	S	I	V	D	P	N	R	M	G	V	V	T	F	Q	A	F	I	D	F	M	S	R	E	13	1693	44
FIB	H	N	M	G	Q	F	S	T	W	D	N	D	N	D	K	F	E	G	N	C	A	E	Q	D	G	S	G	W	W	6	1542	42

Fig. 8. **a:** Comparison of known PKC phosphorylation sites to potential sites in pleckstrin. Sequences of eight PKC substrates were aligned four at a time using a window size of ten residues and a matrix which favored alignment of basic residues and alignment of serine or threonine residues. The best alignment of these—interleukin-2 receptor (IL-2-R), smooth muscle myosin light chain (SM-MLC), ribosomal S6 protein (RS6), and PKC pseudosubstrate (PKCPS)—was used as a core set for comparison to other substrate sequences—myelin basic protein (MBP), epidermal growth factor receptor (EGFR), $p60^{src}$ (P60), and p36 (P36)—and the entire pleckstrin (PLE) protein sequence. Align scores for each sequence are indicated. Phosphorylated sites in known substrates and putative sites in pleckstrin are boxed, while basic residues are underlined. **b:** Comparison of predicted pleckstrin EF-hand to similar structures in known Ca^{2+} binding proteins: i) numbering system proposed by Kretsinger [21]; ii) consensus sequence (h = A,F,I,L,M,V or Y; d = D,E,N,Q,S, or T; i = I or V); iii) octahedral vertices formed by liganding oxygens; PLE, pleckstrin (292–320); CaM, calmodulin (I 19–37); S100 (β52–80); p11 (50–78); αACT, chicken fibroblast α-actinin (786–814); FIB, fibrinogen (γ307–336). The degree of similarity, based on the criteria of Kretsinger [21] (KI) and a modified multiple sequence alignment program [20] (ALN) is shown by scores on the right.

In summary, we have cloned and partially characterized the cDNA for pleckstrin, a unique hemopoietic protein and the major PKC substrate of platelets. We are pursuing the isolation of genomic clones to help characterize this complex transcriptional unit and are also testing both platelet and recombinant pleckstrin for Ca^{2+}-binding and actin-regulating activity.

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