65-Kilodalton Protein Phosphorylated by Interleukin 2 Stimulation Bears Two Putative Actin-Binding Sites and Two Calcium-Binding Sites[†,‡]

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ABSTRACT: We have previously characterized a 65-kilodalton protein (p65) as an interleukin 2 stimulated phosphoprotein in human T cells and showed that three endopeptide sequences of p65 are present in the sequence of l-plastin [Zu et al. (1990) *Biochemistry 29*, 1055–1062]. In this paper, we present the complete primary structure of p65 based on the cDNA isolated from a human T lymphocyte (KUT-2) cDNA library. Analysis of p65 sequences and the amino acid composition of cleaved p65 N-terminal peptide indicated that the deduced p65 amino acid sequence exactly coincides with that of l-plastin over the C-terminal 580 residues [Lin et al. (1988) *Mol. Cell. Biol. 8*, 4659–4668] and has a 57-residue extension at the N-terminus to l-plastin. Computer-assisted structural analysis revealed that p65 is a multidomain molecule involving at least three intriguing functional domains: two putative calcium-binding sites along the N-terminal 80 amino acid residues; a putative calmodulin-binding site following the calcium-binding region; and two tandem repeats of putative actin-binding domains in its middle and C-terminal parts, each containing approximately 240 amino acid residues. These results suggest that p65 belongs to actin-binding proteins.

A 65-kilodalton protein (p65) has been characterized as an interleukin 2 stimulated phosphoprotein in human T cells (Zu et al., 1990). When cell lines dependent on interleukin 2 (IL 2)[1] were treated with this lymphokine, the phosphorylation of five proteins including p65 can be detected by two-dimensional gel electrophoretic analysis. After cell conversion from an IL 2 dependent state to an IL 2 independent state, p65 becomes constitutively phosphorylated to a high extent even without addition of IL 2. Similarly, the constitutive phosphorylation of p65 occurs without IL 2 stimulation in other IL 2 independent T cell lines as well. In contrast, the phosphorylation of the other four proteins remains to be induced by IL 2 either in the IL 2 independent variant or in its IL 2 dependent parent. Thus, it seems that the increased phosphorylation of p65 is related to cellular growth more closely than that of the others. Previously we have purified p65 to homogeneity and determined its partial amino acid sequence for three internal peptides (Zu et al., 1990). A computer-assisted search for protein sequence homology with reported protein sequences revealed that all these peptides exist in l-plastin, which was originally identified as an abundant transformation-induced protein in chemically transformed human fibroblasts (Lin et al., 1988). On the other hand, Matsushima et al. (1987) identified a cytosol 65-kDa protein in glucocorticoid-pretreated human peripheral blood mononuclear cells whose serine phosphorylation was rapidly induced by IL 1. They purified the protein from human leukocytes and determined its partial amino acid sequence (Matsushima et al., 1988), which has been suggested to be identical with l-plastin (Lin et al., 1988). Thus, it is now evident that the serine phosphorylation of a common cytosol protein, p65 plausibly identical with l-plastin, is involved in the intracellular signaling pathways of IL 1 and IL 2.

We further noticed that the amino acid composition of p65 is very similar to that of an actin-binding protein, acumentin (Southwick & Stossel, 1981). In addition, both of them exist in cytosol of leukocytes in abundance and have a similar molecular weight of about 65 000. Therefore, it is highly probable that p65 and acumentin are the same protein, although no amino acid sequence information of acumentin has been reported yet. The enhanced phosphorylation of some cytoskeletal proteins has been recently regarded as an essential process in transmitting the initial transmembrane signal for cell growth. For example, phosphorylation of vinculin (Werth & Pastan, 1984), myosin light chain (Bockus & Stiles, 1984), and microtubule-associated protein (MAP-1) (Sato et al., 1985) have been reported as early events after stimulation of cells by phorbol esters or growth factors.

In this paper, we present the complete primary structure of p65 based on the cDNA isolated from a human T lymphocyte cDNA library. Computer-assisted structural analysis revealed that p65 is a multidomain molecule involving at least three intriguing functions: two N-terminal calcium-binding sites; a calmodulin-binding site following the calcium-binding region; and two actin-binding regions in its middle and C-

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[1] Abbreviations: DTT, dithiothreitol; HPLC, high-performance liquid chromatography; IL 2, interleukin 2; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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terminal parts. These results disclose the novel functional potentials of p65.

Materials and Methods

Construction of a cDNA Library. A human T cell line, KUT-2, was used for cloning p65 cDNA, because the cells contain a highly abundant p65 (Zu et al., 1990) and can grow well without any growth factors. Total RNAs were prepared from cultured cells by the guanidine thiocyanate method (Chirgwin et al., 1979). Poly(A+) RNA was isolated by two cycles on an oligo(dT)–cellulose column (Pharmacia Fine Chemicals, Piscataway, NJ). The cDNA library was constructed by the modified method of Okayama and Berg (1982) using vector pSI4001 (Shigesada et al., 1987; Kawamura et al., 1990). In brief, the first-strand cDNA was synthesized with a dT-tailed vector DNA fragment as a primer. The resultant cDNA/mRNA vector was converted to a linear double-stranded DNA with flush ends (Gubler & Hoffman, 1983). Then the cDNA vector was directly circularized by T4 DNA ligase in the presence of 1 mM hexamminecobalt, an agent known to facilitate self-ligation of blunt-ended DNA (Rusche & Howard-Flanders, 1985). This procedure is very rapid, technically easy, and yet equivalent in its overall efficiency to the original Okayama–Berg method (Okayama et al., 1987). The DNA was transformed into *Escherichia coli* strain DH-1 as described by Maniatis et al. (1982) to yield a cDNA library consisting of about 1×10^6 independent clones.

Screening of the cDNA Library. We have determined three internal peptide sequences of p65, which coincided with the sequence for l-plastin. Thus, we synthesized the mixed oligonucleotides corresponding to the part of the l-plastin amino acid sequence from residues 323 to 329 (Figure 2): 5′-GAI-AA(TC)-CAI-GA(TC)-AT(TCA)-GA(TC)-TGG-3′, in which I's represent inosine residues. Then the oligonucleotides were labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP and used as probes. The cDNA library was spread on culture plates to give about 2000 colonies per plate and replicated onto nitrocellulose filters (Millipore Triton-free HATF), which were then screened by hybridization with the ^{32}P-labeled probes. Hybridizations were performed at 37 °C overnight in a buffer solution consisting of 6 × SSC (1 × SSC contains 15 mM sodium citrate and 150 mM NaCl), 1 × Denhardt's solution [0.2% poly(vinylpyrrolidone), 0.2% Ficoll 400, and 0.2% bovine serum albumin], 0.1% SDS, and 0.05% pyrophosphate. Filters were washed at 47 °C and at 49 °C in 6 × SSC containing 0.05% pyrophosphate for 1 and 2 h, respectively. Screening of about 2×10^5 colonies yielded 5 positive clones. The positive clones were isolated and purified to homogeneity.

DNA Sequencing and Subjection to Restructure Endonuclease Mapping. The longest cDNA insert from CLONE 5 was subcloned into Bluescript KS+ and SK− vectors: a 1.2-kb *Eco*RI–*Sph*I fragment encompassing the N-terminal one-third and a small vector-derived segment and a 2.4-kb *Sph*I–*Eco*RI fragment containing most of the remaining part of the insert. The subcloned plasmids were digested with *Kpn*I and *Sac*I to protect the vector and with *Eco*RI and *Bam*HI to allow digestion into the inserts by ExoIII exonuclease for 1.2- and 2.4-kb subclones respectively. Nested deletions were constructed according to the procedure supplied with the Bluescript vectors (see Figure 1). Sequencing of the subcloned DNAs was performed by Sanger's dideoxy chain termination method (Sanger et al., 1980) with M13 universal primer and reverse primer, modified T7 DNA polymerase, and dITP in place of dGTP using a sequencing kit from United States Biochemical Corp. (Cleveland, OH).

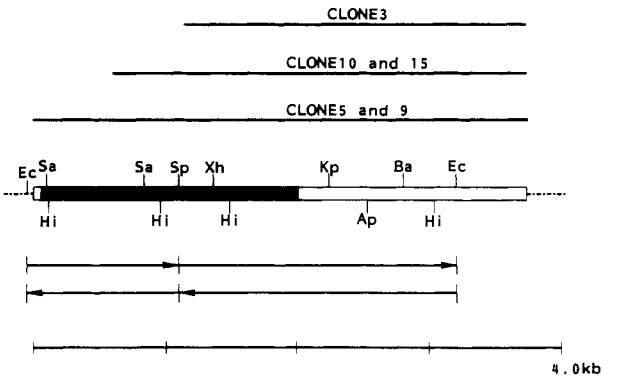


FIGURE 1: Restriction enzyme map and sequencing strategy for the p65 cDNA. The mapped restriction sites are *Apa*I (Ap), *Bam*HI (Ba), *Eco*RI (Ec), *Hin*dIII (Hi), *Kpn*I (Kp), *Sac*I (Sa), *Sph*I (Sp), and *Xho*I (Xh). The dideoxy chain termination method (Sanger et al., 1980) was used to sequence CLONE 5 which was divided in two halves at the unique *Sph*I site within the cDNA. Arrows indicate the sequenced regions for both strands. The putative protein-coding region is indicated by a solid box and the 5′- and 3′-untranslated region by open boxes. The dotted line indicates the flanking vector sequence.

Amino Acid Analysis of Cleaved p65 Amino-Terminal Peptide Fragment. The purified p65 (from Jurkat cells) was cleaved by lysyl endopeptidase, and the cleaved peptides were separated by reverse-phase HPLC as described previously (Zu et al., 1990). The amino-terminal peptide of p65 (p11) was further purified by rechromatography under a different condition (low trifluoroacetic acid concentration, 0.01%). The amino acid composition of this peptide fragment was determined in duplicate on a Hitachi Model L-8500 amino acid analyzer after hydrolysis in 6 N HCl for 24 h at 110 °C in vacuo.

Computer Analysis of the Sequences. The sequence was analyzed by using the program package GENETYX (SDC Software Development Co., Tokyo). Searches for similarities to other DNA or protein sequences were done with the programs Fastn and Fastp (Lipman & Pearson, 1985) using the databanks GenBank and NRBF as installed in a compact disc system from SDS Software Development Co.

Results

Isolation of cDNA Clones. Human T cells, KUT-2, were chosen for the construction of the cDNA library because they showed relatively good growth without any exogenous growth factors and a high content of p65, favorably comparing to other cell lines examined. After determining that the sequences of three oligopeptides of p65 exist in the sequence of l-plastin, we used mixed oligonucleotides as probe. Using a ^{32}P-labeled mixture of oligonucleotides (24×) synthesized according to a partial amino acid sequence of l-plastin (see Materials and Methods and Figure 2), we isolated 5 positive cDNA clones from about 2×10^5 colonies. Upon restriction endonuclease digestion, cDNA inserts of those gave overlapping cleavage patterns, suggesting that they were derived from an identical mRNA species. The largest insert found in CLONE 5 was 3.7 kb in length, which is equivalent to the reported length of l-plastin mRNA. The sequence of CLONE 5 was determined from its 5′ end to the restriction *Eco*RI site located within the 3′-terminal untranslated region (Figure 1).

Analysis of p65 Sequences. In the deduced amino acid sequence (Figure 2), there are four methionine codons at the N-terminal region (Figure 3A) including the reported initiation position for l-plastin (the fourth methionine). Among these methionine codons, only the first and the third codons are surrounded by nucleotide bases corresponding to the consensus

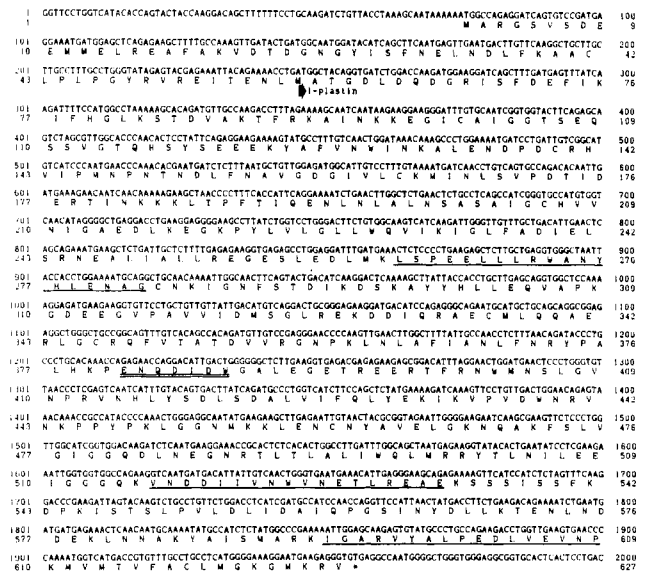


FIGURE 2: Complete nucleotide and amino acid sequences derived from the p65 cDNA CLONE 5. The amino acid sequence for the longest open reading frame from the first putative translation initiation signal is shown with single-letter codes. Numbering of the amino acid sequence begins at the postulated initiator methionine. The nucleotide sequence is numbered from the 5′ end of the cDNA insert. The determined three internal peptide sequences of p65 are underlined. The sequence corresponding to the probe oligonucleotides used for screening is marked by double lines. The arrow indicates the reported initiation position of l-plastin (Lin et al., 1988).

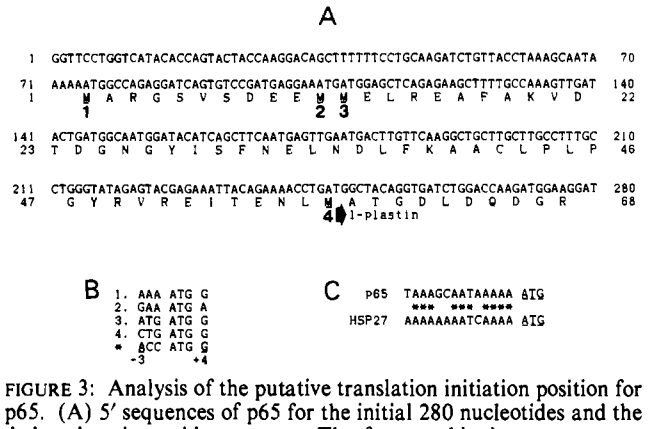


FIGURE 3: Analysis of the putative translation initiation position for p65. (A) 5′ sequences of p65 for the initial 280 nucleotides and the deduced amino acid sequence. The four methionines present are numbered. The arrow indicates the reported putative initiation position of l-plastin. (B) Comparison of the putative initiation sequences surrounding the four ATG found above with the consensus sequence (asterisk) for initiation by eukaryotic ribosomes (Kozak, 1986). (C) Comparison of the sequence immediately preceding the first putative initiation codon of p65 with those of heat-shock 27-kDa protein (HSP27) (Southgate et al., 1983). Asterisks indicate A residues matching between flanking pairs.

sequence for initiation of eukaryotic translation (Kozak, 1986): the strong preference for a purine, most often A, three nucleotides upstream from the AUG initiation codon (−3′) and the preference for G in the position immediately after the AUG (+4′) as seen in Figure 3B. The second methionine codon with an A at the +4′ position and the fourth, putative initiation position for l-plastin with a C at the −3′ position are not favorable candidates for initiation by eukaryotic ribosomes. Thus, it seems that the first methionine is the most likely initiation position for p65 translation (Figure 3A). Notable

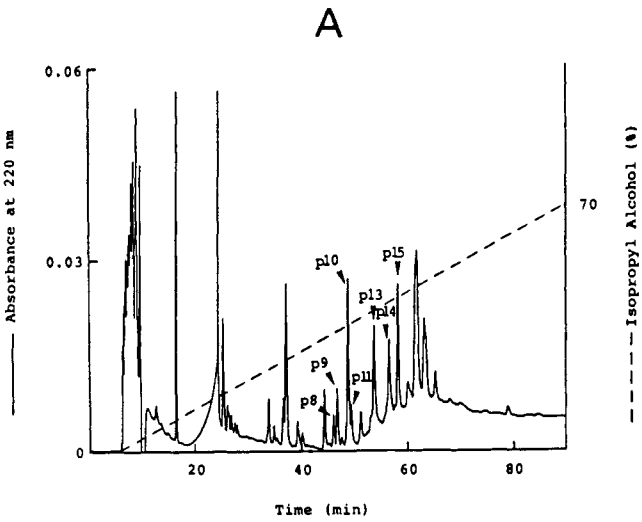
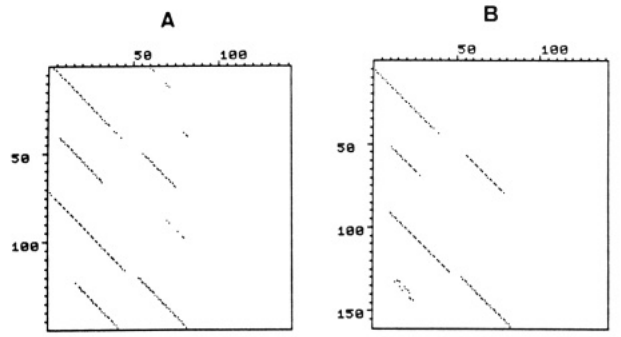


FIGURE 4: Analysis of the amino acid composition for cleaved p65 N-terminal peptide. The purified p65 was digested and separated by reverse-phase HPLC (A); the arrows indicate the fragments which were sequenced. (B) The top row shows the amino acid sequence of the predicted p65 N-terminal peptide cleaved by lysyl endopeptidase. The determined amount of amino acid residues of peptide 11 was shown and compared with the deduced number of amino acid residues in the p65 N-terminal peptide (top row) in parentheses.

in this regard is that the A-rich sequence of p65 preceding the first methionine is very similar to that of HSP27 (Southgate et al., 1983) as shown in Figure 3C.

Because the N-terminus of p65 was blocked, we attempted to determine the amino acid composition of the cleaved p65 N-terminal peptide. The purified p65 was digested with lysyl endopeptidase, the resultant peptides were separated by reverse-phase HPLC (Figure 4A), and their amino acid sequences were determined. Among them, one peptide, p11, could not be sequenced because of its N-terminus block suggesting it to be p65 N-terminal peptide. This peptide was further purified by HPLC under low TFA (trifluoroacetic acid) concentration (0.01%). Under such a condition, p11 was well separated from p10 (its amino acid sequence is YAFVNWINK, residues 124–132). The amino acid composition of the purified p11 was determined and compared to that of the cleaved p65 N-terminal peptide (Figure 4B). This result strongly suggests that p65 is translated from the first methionine. In reduction and alkylation of proteins, the methionine residues are often alkylated to some extent. That may be the reason why the calibrated amount of methionine residue is considerably low as compared with the calculated number of methionine residues. With this putative initiation codon, the deduced protein sequence exactly coincides with that of l-plastin over the C-terminal 580 residues and has a 57 amino acid extension N-terminal to the l-plastin sequence



C

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                EL   LL   LD D DG ID   EL   LL   L
p65 I   10  EMMELREAFAKVDTDGNGYISFNELNDLFKAACL   33
p65II   50  VREITENLMATGDLDQDGRISFDEFIKIFHGLKS   83
HC      81  SEEEIREAFRVFDKDGNGYISAAELRHVMTNLGE  114
HT      51  TKEELDAIIEEVDEDGSGTIDFEEFLVMMVRQMK   84
RP      39  SDAELAEIFNVLDGDQSGYIEVEELKNFLKCFSD   72
DA     767  TEEQLNQVISKIDTDGNGTISFEEFIDYMVSSRK  800
                X  Y  Z -Y -X  -Z
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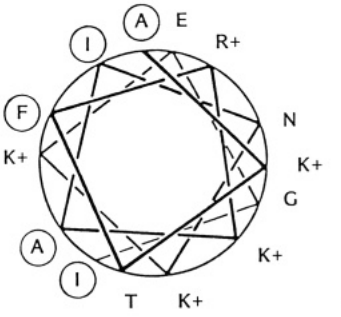
FIGURE 5: Homology search of p65 with calcium-binding proteins. Dot plot analysis of the p65 N-terminal sequence with human calmodulin (Sasagawa et al., 1982) and human troponin C (Gahlmann et al., 1988) was performed using Harplot (GENETYX, SDS Software Development Co., Tokyo). The comparisons of p65 with calmodulin (A) and with troponin C (B) show a similarly strong homology between all three proteins in their putative calcium-binding regions. The x axis indicates the p65 N-terminal region, and the y axis indicates calmodulin and troponin C with their amino acid sequence numbered. (C) Alignment of the putative EF-hand regions of p65 and known calcium-modulated proteins: HC, human calmodulin; HT, human troponin; RP, *Raja clavata* parvalbumin (Thatcher & Pechere, 1977); DA, *Dictyostelium discoideum* α-actinin (Noegel et al., 1987). According to the EF-hand hypothesis (Kretsinger & Nockolds, 1973), there are 16 characteristic positions (top row) building an EF-hand structure. A true EF-hand domain scores 12 or better out of 16. The boxed residues indicate the correct alignment and the liganding oxygens that correspond to the octahedral vertices (bottom row). Conserved amino acid residues are shadowed. The numbers indicate the leftmost amino acid position of each sequence.

(Figure 2). In total, the deduced amino acid sequence of p65 is 627 residues long, and its calculated molecular weight is 70 306, which is a little larger than the apparent molecular weight (65K) estimated from SDS–polyacrylamide gel electrophoresis.

Potential Functional Domains in p65. Systematic search with protein databanks for sequences that are homologous to p65 indicated three functionally interesting regions in p65. First, the N-terminal sequence of about 80 amino acid residues has a strong homology to that of calcium-binding proteins, showing the best matches to calmodulin among them. Panels A and B of Figure 5 show a dot plot analysis that compares the N-terminal sequence of p65 with that of calmodulin (Sasagawa et al., 1982) and troponin C (Gahlmann et al., 1988), respectively. These putative calcium-binding sequences are further compared with the corresponding regions of these and other known calcium-binding proteins (Figure 5C). In this region, there are two so-called EF-hands (i.e., Ca^{2+}-binding loops). Second, by visual inspection, we noticed a unique sequence (residues 87–100) that contains clusters of basic and hydrophobic residues immediately downstream from the putative calcium-binding region. By these criteria, it might represent a calmodulin-binding site (Kemp et al., 1987). Helical wheel analysis (Figure 6) reveals its amphipathic nature and net positive charges, which are the consensus drawn for calmodulin-binding sites in proteins (Erickson-Viitanen & De Grado, 1987). Third, dot plot comparison of p65 with *Dictyostelium discoideum* α-actinin (Figure 7A) (Noegel et al., 1986) and chick α-actinin (Figure 7B) (Baron et al., 1987)



87' AKTFRKAINKKEGI 100'

FIGURE 6: Helical wheel presentation of the putative calmodulin-binding site spanning residues 87–100 in p65. Hydrophobic residues are circled and (+) indicates basic residues.

showed a clear homology involving about 240 amino acid residues at the N-terminus of α-actinin. This region has been reported to exhibit a monovalent and Ca^{2+}-insensitive actin-binding activity (Mimura & Asano, 1987) and found to be conserved in *D. discoideum* α-actin, chick fibroblast α-actinin, human dystrophin (Noegel et al., 1987), and *D. discoideum* gelation factor (Noegel et al., 1989). The homology search over p65 itself (Figure 7C) further shows that p65 has 2 strong repeated regions of approximately 240 amino acid residues in the middle and C-terminal regions, each being homologus to actin-binding domains of α-actinin. Figure 7D compares the core region of homology for p65 and 4 known actin-binding proteins over about 40 amino acid residues.

In summary, along the N-terminal 80 amino acid residues of p65, there are 2 EF-hand Ca^{2+}-binding sites. Following this sequence is a segment that could be a calmodulin-binding site from 87′ to 100′. Then, at the middle and C-terminal regions are two tandem repeats of putative actin-binding domains, making a sharp contrast to other actin-binding proteins reported so far, which contain either only one actin-binding site (α-actinin; Mimura & Asano, 1987) or two interrupted actin-binding sites (gelsolin; Kwiatkowski et al., 1985) as shown in Figure 8. These actin-binding domains of p65 showed clear homology to that of α-actinin, but not to those of gelsolin. Therefore, it is suggested that p65 is a novel member of the actin-binding protein family and should have some unique functions different from those known for actin-binding proteins. In preliminary in vitro experiments, the purified p65 inhibited the polymerization of G-actin obtained from rabbit skeletal muscle (data not shown). The purified p65 showed a clear inhibitory effect at fairly low concentration (approximately 50% inhibition by 0.5 μM p65 added to 4 μM actin solution).

Discussion

We present here the nucleotide sequence covering the entire coding frame and the 5′-flanking sequence plus a part of the 3′-untranslated region of the mRNA for the 65-kilodalton protein. The mRNA which was used as a template in the cDNA synthesis was isolated from a human T lymphocyte cell line, KUT-2. The full length of p65 mRNA is about 3750 bp, in which the 3175 bp nucleotides from 5′ have been sequenced thus far. Since all three determined oligopeptide sequences of p65 are present in the deduced p65 amino acid sequence, it is evident that this cDNA represents p65. The deduced amino acid sequence has four methionine codons at its N-terminalmost region. Of these, the first methionine codon is flanked by a favorable consensus sequence for initiation of translation defined by Kozak (1986). From the

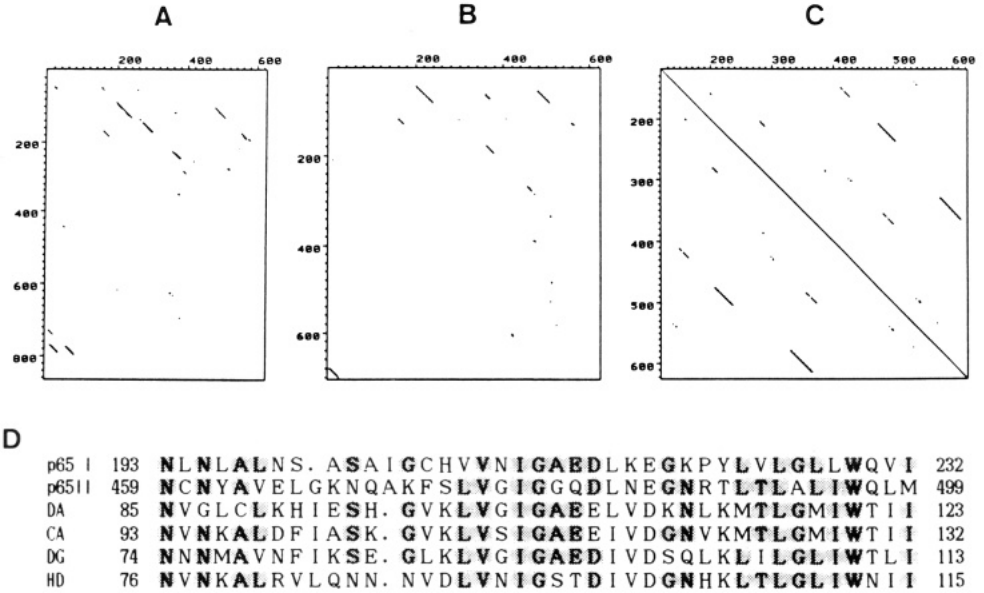


FIGURE 7: Homology search of p65 with known actin-binding proteins. Dot plot analysis of p65 with *Dictyostelium discoideum* α-actinin (A), with chick α-actinin (B), and with itself (C) was performed as in Figure 5. The *x* axis represents p65, and the *y* axis represents actinins with their amino acid numbered. (D) Alignment of the core regions of homology found in p65 and *D. discoideum* α-actinin (DA) (Noegel et al., 1986), chick α-actinin (CA) (Baron et al., 1987), *D. discoideum* gelation factor (DG) (Noegel et al., 1989), and human dystrophin (HD) (Koenig et al., 1988). Identical residues which are shared by at least four proteins are shadowed. The numbers on both sides of each sequence indicate the terminal residue position.

FIGURE 8: Predicted domain structure of p65. Three main functional domains of p65 are indicated: circles, 2 putative calcium-binding sites in the first 80 amino acid residues; square box, a potential calmodulin-binding site spanning amino acid residues 87–100; solid bars, putative actin-binding sites homologous to α-actinin spanning amino acid residues 140 to end.

amino acid composition analysis of cleaved p65 N-terminal peptide, we may conclude that the first methionine is the initiation position for p65 translation. The deduced p65 sequence has 627 amino acid residues and is 57 amino acid residues longer than that of l-plastin on the N-terminal end. It seems possible that the reported l-plastin clone was not complete. Residues 1–80 contain two potential EF-hand Ca^{2+}-binding sites; both of them are strongly homologous to the calcium-binding sites in calmodulin (Sasagawa et al., 1982) and retain all conserved oxygen-containing amino acid side chains known to be essential for Ca^{2+} chelating (Kretsinger & Nockolds, 1973). Thus, these sites should be functional, and hence p65 is likely to be a nonmuscle, calcium-controlled protein. Following this region is a putative calmodulin-binding site in a clusters of 16 basic and hydrophobic residues. The structure of successive calcium-binding sites and a calmodulin-binding site is similar to that of α-spectrin (Wasenius et al., 1989) though being arranged in the opposite order. At the middle and C-terminal regions are 2 continuous repeats of putative actin-binding domains, each being composed of about 240 amino acid residues and homologous to the actin-binding site in α-actinin (Noegel et al., 1986). It has been suggested that the conserved sequence of about 240 amino acid residues in the actinin family is of critical importance for the actin-binding activity (Mimura & Asano, 1987). Judging from this structure, p65 is presumably a divalent actin-binding protein. The amino acid composition of p65 is very similar to that of an actin-binding protein, acumentin (Southwick & Stossel, 1981) Acumentin has been reported to exist in the cytosol of leukocytes in abundance and inhibit the polymerization of actin. Our preliminary experimental results indicate that p65 inhibits polymerization of α-actin as acumentin does. Therefore, it is highly probable that p65 and acumentin are the same protein. However, the detailed nature of the interaction of p65 with actin must be studied fully in future work, in which effects of calcium and calmodulin on the interaction should also be examined. It is also very intriguing to investigate the effect of phosphorylation of p65 on its actin-interacting activity. The serine phosphorylation of p65 can be rapidly induced by some cellular growth factors, such as IL 1 in glucocorticoid-pretreated human peripheral blood mononuclear cells (Matsushima et al., 1987) and IL 2 and TPA in human T lymphocytes (Zu et al., 1990). The enhanced phosphorylation of some cytoskeletal proteins by stimulation of growth factors has been recently considered to correlate with cellular growth. Thus, the phosphorylation of p65 as the factor controlling cytoskeletons might be involved in the intracellular signaling pathways. The serine residue of p65 which is phosphorylated by IL 2 stimulation exists in the first EF-hand Ca^{2+}-binding site (unpublished data), suggesting that the phosphorylation of this serine residue might change the calcium dependency of p65.

The present study with a complete cDNA for p65 revealed its multiple structural elements which may carry some important functions, thus opening a new way for further study of the molecular mechanism and regulatory role of p65. It seems important to clarify how the phosphorylation of p65 is related to the cellular proliferation.

While this paper was in preparation, J. Leavitt kindly sent us the galley proof of their paper concerning the structure of l-plastin (Lin et al., 1990). In this paper, they obtained cDNA of l-plastin which is longer than what they have previously reported. Using this cDNA, they demonstrated that the amino acid sequence of l-plastin continues further upstream from the sequence that they previously reported. They have also obtained evidence that the first calcium-binding site exists in l-plastin by microsequencing of the enzyme-digested peptide of l-plastin.

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References

Baron, M. D., Davison, M. D., Jones, P., & Critchley, D. R. (1987) *J. Biol. Chem. 262*, 17623–17629.

Bockus, B. J., & Stiles, C. D. (1984) *Exp. Cell Res. 153*, 186–197.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 533–543.

Erickson-Viitanen, S., & De Grado, W. F. (1987) *Methods Enzymol. 139*, 455–478.

Gahlmann, R., Wade, R., Gunning, R., & Kedes, L. (1988) *J. Mol. Biol. 20*, 379–391.

Gubler, U., & Hoffman, B. J. (1983) *Gene 25*, 263–269.

Kawamura, T., Shigesada, K., Yanagisawa, S., & Izui, K. (1990) *J. Biochem. 107*, 165–168.

Kemp, B. E., Pearson, R. B., Guerriero, V., Jr., Bagchi, I. C., & Means, A. R. (1987) *J. Biol. Chem. 262*, 2542–2548.

Koenig, M., Monaco, A. P., & Kunkel, L. M. (1988) *Cell 53*, 219–228.

Kozak, M. (1986) *Cell 44*, 283–292.

Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248*, 3313–3326.

Kwiatkowski, D. J., Janmey, P. A., Mole, J. E., & Yin, H. L. (1985) *J. Biol. Chem. 260*, 15232–15238.

Lin, C.-S., Aebersold, R. H., Kent, S. B., Varma, M., & Leavitt, J. (1988) *Mol. Cell. Biol. 8*, 4659–4668.

Lin, C.-S., Aebersold, R. H., & Leavitt, J. (1990) *Mol. Cell. Biol.* (in press).

Lipman, D. J., & Pearson, W. R. (1985) *Science 227*, 1435–1441.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 254–255.

Matsushima, K., Kobayashi, Y., Copeland, T. D., Akahoshi, T., & Oppenheim, J. J. (1987) *J. Immunol. 139*, 3367–3374.

Matsushima, K., Shiroo, M., Kung, H., & Copeland, T. D. (1988) *Biochemistry 27*, 3765–3770.

Mimura, N., & Asano, A. (1987) *J. Biol. Chem. 262*, 4717–4723.

Noegel, A., Witke, W., & Schleicher, M. (1986) *FEBS Lett. 204*, 107–109.

Noegel, A., Witke, W., & Schleicher, M. (1987) *FEBS Lett. 221*, 391–396.

Noegel, A., Rapp, S., Lottspeich, F., Schleicher, M., & Stewart, M. (1989) *J. Cell Biol. 109*, 607–618.

Okayama, H., & Berg, P. (1982) *Mol. Cell. Biol. 3*, 280–289.

Okayama, H., Kawichi, M., Brownstein, M., Lee, F., Yokota, T., & Arai, K. (1987) *Methods Enzymol. 154*, 3–28.

Rusche, J. R., & Howard-Flanders, P. (1985) *Nucleic Acids Res. 13*, 1997–2008.

Sanger, F., Coulsen, A. R., Barrell, B. G., Smith, A. J. H., & Roe, B. (1980) *J. Mol. Biol. 143*, 161–178.

Sasagawa, T., Ericsson, L. H., Walsh, K. A., Schreiber, W. E., Fischer, E. H., & Titani, K. (1982) *Biochemistry 21*, 2565–2569.

Sato, C., Nishizawa, K., Nakayama, T., & Kobayashi, T. (1985) *J. Cell Biol. 100*, 748–753.

Shigesada, K., Itamura, S., Kato, M., Hatanaka, M., Imai, M, Tanaka, M., Masuda, N., Nagai, J., & Nakashima, K. (1987) *Gene 53*, 163–172.

Southgate, R., Ayme, A., & Voellmy, R. (1983) *J. Mol. Biol. 165*, 35–57.

Southwick, F. S., & Stossel, T. P. (1981) *J. Biol. Chem. 256*, 3030–3036.

Thatcher, D. R., & Pechere, J.-F. (1977) *Eur. J. Biochem. 75*, 121–132.

Wasenius, V.-M., Saraste, M., Salven, P., Eramaa, M., Holm, L., & Lehto, V.-P. (1989) *J. Cell Biol. 108*, 79–93.

Werth, D. K., & Pastan, I. (1984) *J. Biol. Chem. 259*, 5264–5270.

Zu, Y., Kohno, M., Kubota, I., Nishida, E., Hanaoka, M., & Namba, Y. (1990) *Biochemistry 29*, 1055–1062.