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The primary structure of the β-subunit of the cell surface adhesion glycoproteins LFA-1, CR3 and p150,95 and its relationship to the fibronectin receptor

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The lymphocyte-function-associated antigen-1 (LFA-1), the complement receptor type 3 (CR3) and the antigen p150,95 are cell-surface glycoproteins. They are heterodimeric complexes, each containing a unique α-subunit noncovalently associated with a common β-subunit. We have purified the β-subunit from human spleen and obtained limited peptide sequences. What appears to be the complete primary structure for the fully processed β-subunit was obtained by cDNA sequencing of clones from a phorbol ester (PMA) stimulated U937 cDNA library. There are five possible glycosylation sites and a transmembrane segment. The sequence contains a high level of cysteine (7.6%), with 24 of the 57 cysteine residues being found in three repeating units each with eight residues. The entire primary structure has 47% identity to a subunit of a fibronectin binding protein from chicken fibroblasts. It seems that LFA-1, CR3 and p150,95 antigens may belong to an extended family of cell surface molecules including the fibronectin binding protein.

Key words: β-subunit/CR3, LFA-1, p150,95/fibronectin receptors

Introduction

The lymphocyte-function-associated antigen-1 (LFA-1), the complement receptor type 3 (CR3) and the antigen p150,95 are cell-surface glycoprotein complexes each containing a unique α-subunit and presumably an identical β-subunit (Sanchez-Madrid *et al.*, 1983). The apparent mol. wts of the α-subunits, as determined by SDS–PAGE, are 175 000, 165 000 and 150 000 daltons respectively, and that of the β-subunit is 95 000 daltons. Each of the three complexes participates in some form of cell-adhesion activities; LFA-1 mediates interactions between T-cells and their targets (Sanchez-Madrid *et al.*, 1982; Hildreth *et al.*, 1983); CR3 has affinity for iC3b, a natural degradation product of C3 by factors I and H in the complement system (Ross *et al.*, 1983) and the interaction may promote phagocytic activity (Wright and Silverstein, 1982, 1983); p150,95 has a similar binding specificity to CR3 but the resultant manifestation of this interaction is not known (Micklem and Sim, 1985; Malhotra *et al.*, 1986).

Patients deficient in this group of glycoproteins suffer from recurrent bacterial and fungal infections (Anderson *et al.*, 1985; Arnaout *et al.*, 1985; Springer *et al.*, 1985a). Invariably, they are deficient in all three complexes suggesting that the deficiency is in the common β-subunit. Furthermore, it is apparent that the absence of the β-subunit interferes with the maturation and expression of the α-subunits (Springer *et al.*, 1985a).

Recently, it has been speculated that this group of adhesion proteins may belong to a more extended family of cell-surface proteins including the fibronectin receptor (Tamkun *et al.*, 1986), the vitronectin receptor (Suzuki *et al.*, 1986), and the gpIIb/IIIa glycoprotein of platelets (Cosgrove *et al.*, 1986; Pytela *et al.*, 1986; Pierce *et al.*, 1987), all of which have specific affinity for peptides containing the amino acid sequence Arg Gly Asp (RGD) as found in the binding region of the fibronectin molecule (Pytela *et al.*, 1985; Pierschbacher and Ruoslahti, 1984; Wright and Meyer, 1985). Such speculation is reinforced by the observation that a synthetic peptide from C3, which contains the sequence RGD, can bind to CR3 (Wright *et al.*, 1987).

We focused our study on the β-subunit of this set of adhesion glycoproteins with the aim of determining the primary structure by a combination of protein and cDNA sequencing. The β-subunit protein sequence was found to be very similar to chicken integrin, a subunit of a fibronectin binding molecule on fibroblasts (Tamkun *et al.*, 1986).

Results

The purification of the β-subunit of LFA-1

The β-subunit of the cell-surface adhesion glycoproteins was purified from human spleen by affinity chromatography using the H-52 monoclonal antibody (Hildreth and August, 1985). The methodology employed was basically that of Williams and Barclay (1986). The detergent [Nonidet P40 (NP40)] extract of spleen membranes was passed through the H-52 column twice. The bound materials eluted at pH 11.5 in the first passage contained dissociated α- and β-subunits since the interaction between the subunits is irreversibly broken in the alkaline conditions (Sanchez-Madrid *et al.*, 1983). Upon second passage only the β-subunit was bound to the H-52 column and it was eluted at pH 2.2. About 50% of the antigenic activity of the NP40 extract was recovered at this point. The eluted material was succinylated and reduced/alkylated before further purification by gel-filtration through a Sephacryl S-400 column in the presence of 0.2% SDS. About 0.5 mg pf protein, as determined by the method of Lowry *et al.* (1951), was obtained from ~ 70 g of human spleen. The material eluted from the affinity column had an apparent mol. wt of 95 000 daltons on SDS–PAGE and this increased to 120 000 after succinylation and 135 000 daltons apparent mol. wt after succinylation/reduction/alkylation (data not shown).

Tryptic peptides

The succinylated/reduced/alkylated β-subunit was digested with trypsin and the peptides were separated by gel filtration followed by reverse phase h.p.l.c. Twelve of the peptides yielded unique NH_2-terminal sequences. Two peptides, T3-9 and T7-14 contain sequences of CNVCEC and CDGVQI respectively. Two

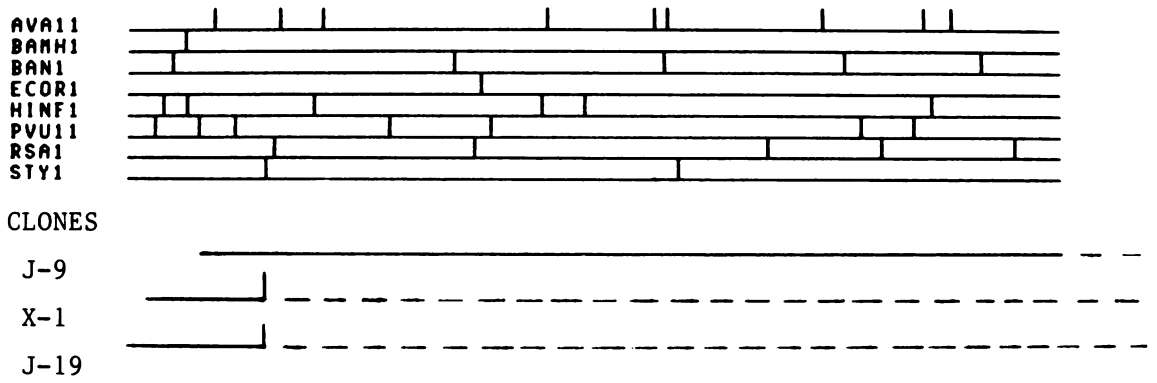

Fig. 1. The restriction maps of clones J-9, X-1 and J-19. The restriction enzyme sites for enzymes used to generate fragments for sequencing are shown. The map was obtained from clone J-9 except for the 5′-extension on X-1 and J-19, where the fragment 5′ of the *Sty*I site was characterised. (----) at the 3′-ends represent segments of the clones that have not been characterised or sequenced.

anti-sense oligonucleotide mixtures, 17 bases in length, and complexity of 64 and 128 respectively, were synthesised according to the sequences.

cDNA cloning

The two oligonucleotide mixtures were used to screen a cDNA library from phorbol ester (PMA) stimulated U-937 cells, which are known to express all three cell surface adhesion glycoproteins (Sanchez-Madrid *et al.*, 1983; Malhotra, 1986). Six positive clones which hybridised to both oligonucleotides were obtained from ~ 35 000 colonies in the first screening. Four of the clones had inserts of ~ 2.7 kb; the other two had inserts of ~ 1.8 kb. The six clones were not duplicates of each other.

One of the clones, J-9, was studied extensively. It contains a poly(A) tail but does not appear to be full length since a Northern blot of the original RNA probed with J-9 showed a single band between 3 and 3.5 kb. The most 5′-end *Sty*I fragment (see restriction maps on Figure 1) was used to rescreen the cDNA library. Two clones, X-1 and J-19, included extra sequence on the 5′-side of J-9. Their restriction maps are also included in Figure 1.

The primary structure of the β-subunit

The primary structure of the β-subunit was determined by a combination of protein and cDNA sequencing. Most of the cDNA sequence was obtained from clone J-9 except for the 5′ end which were from clones X-1 and J-19. The strategy employed was to cleave with restriction enzymes, clone into M13 and sequence clones picked at random (Figure 1). In the later stages, specific fragments were purified to obtain sequences between gaps. The 5′-end sequences of clones X-1 and J-19 were obtained from the fragment 5′ to the first *Sty*I restriction site with and without the *Bam*HI cut in the middle. A poly(A) tail was found at the 3′-end but the clones do not include sequence that could code the leader sequence since no codon for methionine is present. However, an open reading frame is evident and the nucleotide sequence covering this open reading frame is shown in Figure 2. Except for a region from residue numbers 2086–2140 and the 23 bases at the most 3′-end, sequence data from both directions were obtained. In those two regions, the sequence was confirmed from at least three different gel readings. On the average, each position was covered 3.47 times.

The translated protein sequence is shown in Figure 3 together with the positions of the 12 tryptic peptides, which are dispersed throughout the sequence.

Also shown in Figure 3 is the sequence of the integrin molecule, a subunit of the fibronectin binding protein from

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CTCGCCCTCGTGGGGCTCCTCTCCCTCGGGTGCGTCCTCTCTCAGGAGTGCACGAAGTTC    60
AAGGTCAGCAGCTGCCCGGGAATGCATCGAGTCGGGGCCCGGCTGCACCTGGTGCCAGAAG   120
CTGAACTTCACAGGGCCGGGGGATCCTGACTCCATTCGCTGCGACACCCGGCCACAGCTG   180
CTCATGAGGGGCTGTGCGGCTGACGACATCATGGACCCCACAAGCCTCGCTGAAACCCAG   240
GAAGACCACAATGGGGGCCAGAAGCAGCTGTCCCCACAAAAAGTGACGCTTTACCTGCGA   300
CCAGCCCAGGCAGCAGCGTTCAACGTGACCTTCCGCGGGGCCAAGGGCTACCCCATCGAC   360
CTGTACTATCTGATGGACCTCTCCTACTCCATGCTTGATGACCTCAGGAATGTCAAGAAG   420
CTAGGTGGCGACCTGCTCCGGGCCCTCAACGAGATCACCGAGTCCGGCCGCATTGGCTTC   480
GGGTCCTTCGTGGACAAGACCGTGCTGCCGTTCGTGAACACGCACCCTGATAAGCTGCGA   540
AACCCATGCCCCAACAAGGAGAAAGAGTGCCAGCCCCCGTTTGCCTTCAGGCACGTGCTG   600
AAGCTGACCAACAACTCCAACCAGTTTCAGACCGAGGTCGGGAAGCAGCTGATTTCCGCA   660
AACCTGGATGCACCCGAGGGTGGGCTGGACGCCATGATGCAGGTCGCCGCCTGCCCCGAG   720
GAAATCGGCTGCGCCAACGTCACGCCGCTGCTGGTGTTTGCCACTGATGACGGCTTCCAT   780
TTCGCGGGCGACGGAAAGCTGGGCGCCATCCTGACCCCCAACGACGGCCGCTGTCACCTG   840
GAGGACAACTTGTACAAGAGGAGCAACGAATTCGACTACCCATCGGTGGGCCAGCTGGCG   900
CACAAGCTGGCTGAAAACAACATCCAGCCCATCTTCGCGGTGACCAGTAGGATGGTGAAG   960
ACCTACGAGAAACTCACCGAGATCATCCCCAAGTCAGCCGTGGGGGAGCTGTCTGAGGAC  1020
TCCAGCAATGTGGTCCATCTCATTAAGAATGCTTACAATAAACTCTCCTCCAGGGTCTTC  1080
CTGGATCACAACGCCCTCCCCGACACCCTGAAAGTCACCTACGACTCCTTCTGCAGCAAT  1140
GGAGTGACGCACAGGAACCAGCCCAGAGGTGACTGTGATGGCGTGCAGATCAATGTCCCG  1200
ATCACCTTCCAGGTGAAGGTCACGGCCACACAGAGTGCATCCAGGAGCAGTCGTTTGTCATC  1260
CGGGCGCTGGGCTTCACGGACATAGTGACCGTGCAGGTCCTTCCCCAGTGTGAGTGCCGG  1320
TGCCGGGACCAGAGCAGAGACCGCAGCCTCTGCCATGGCAAGGGCTTCTTGCAGTGCGCC  1380
ATCTGCAGGTGTGACACTGGCTACATTGGGAAAAACTGTGAGTGCCAGACACAGGCCCGG  1440
AGCAGCCAGGAGCTGGAAGGAAGCTGCCGGAAGGACAACAACTCCATCATCTGCTCAGGG  1500
CTGGGGGACTGTGTCTGCGGGCAGTGCCTGTGCCACACCAGCGACGTCCCCGGCAAGCTG  1560
ATATACGGGCAGTACTGCGAGTGTGACACCATCAACTGTGAGCGCTACAACGGCCAGGTC  1620
TGCGGCGGCCCCGGGGAGGGGGCTCTGCTTCTGCGGGAAGTGCCCGCTGCCACCCGGGCTTT  1680
GAGGGCTCAGCGTGCCAGTGCGAGAGGACCACTGAGGGCTGCCTGAACCCCCGGCGTGTT  1740
GAGTGTAGTGGTCGTGGCCCGGTGCCGCTGCAACGTATCCGAGTGCCATTCAGGCTACCAG  1800
CTGCCTCTGTGCCAGGAGTGCCCCGGCTGCCCCTCACCCTGTGCCAAGTACATCTCCTGC  1860
GCCGAGTGCCTGAAGTTCGAAAAGGGCCCCTTTGGGAAGAACTGCAGCGCGGCGTGTCCG  1920
GGCCTGCAGCTGTCGAACAACCCCGTGAAGGGCAGGACCTGCAAGGAGAGGGACTCAGAG  1980
GGCTGCTGGGTGGCCTACACGCTGGAGCAGCAGGACGGGATGGACCGCTACCTCATCTAT  2040
GTGGATGAGAGCCGAGAGTGTGTGGCAGGCCCCAACATCGCCGCCATCGTCGGGGGCACC  2100
GTGGCAGGCATCGTCCTCATCGGCATTCTCCTGCTGGTCATCTGGAAGGCTCTGATCCAC  2160
CTGAGCGACCTCCGGGAGTACAGGCGCTTTGAGAAGGAGAAGCTCAAGTCCCAGTGGAAC  2220
AATGATAATCCCCTTTTCAAGAGCGCCACCACGACGGTCATGAACCCCAAGTTTGCTGAG  2280
AGTTAGGAGCA
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Fig. 2. The nucleotide sequence obtained from clones J-9, X-1 and J-19 covering an open reading frame. The first *Sty*I site, starting at position 341, where fragments 5′ to it were obtained and sequenced for clones X-1 and J-19 is marked. Also marked is the termination codon TAG at position 2284.

chicken fibroblasts (Tamkun *et al.*, 1986). Extensive identity is observed between the β-chain of the LFA-1 family of glycoproteins and integrin.

Discussion

A combination of peptide and cDNA sequencing has yielded what appears to be the complete primary structure for the fully processed β-subunit of the cell-surface adhesion glycoproteins LFA-1, CR3 and p150,95.

The protein was purified from human spleen by virtue of its affinity to the monoclonal antibody H-52. This antibody, though initially reported to have specificity for the β-subunit of the three cell-surface glycoprotein complexes (Hildreth and August, 1985), turns out to be more complicated in its interaction with the antigens. It binds to the dissociated β-subunits from all three complexes, but only to the LFA-1 antigen, and not CR3 and p150,95, in their intact α–β forms (Micklem *et al.*, 1986). Hence, the

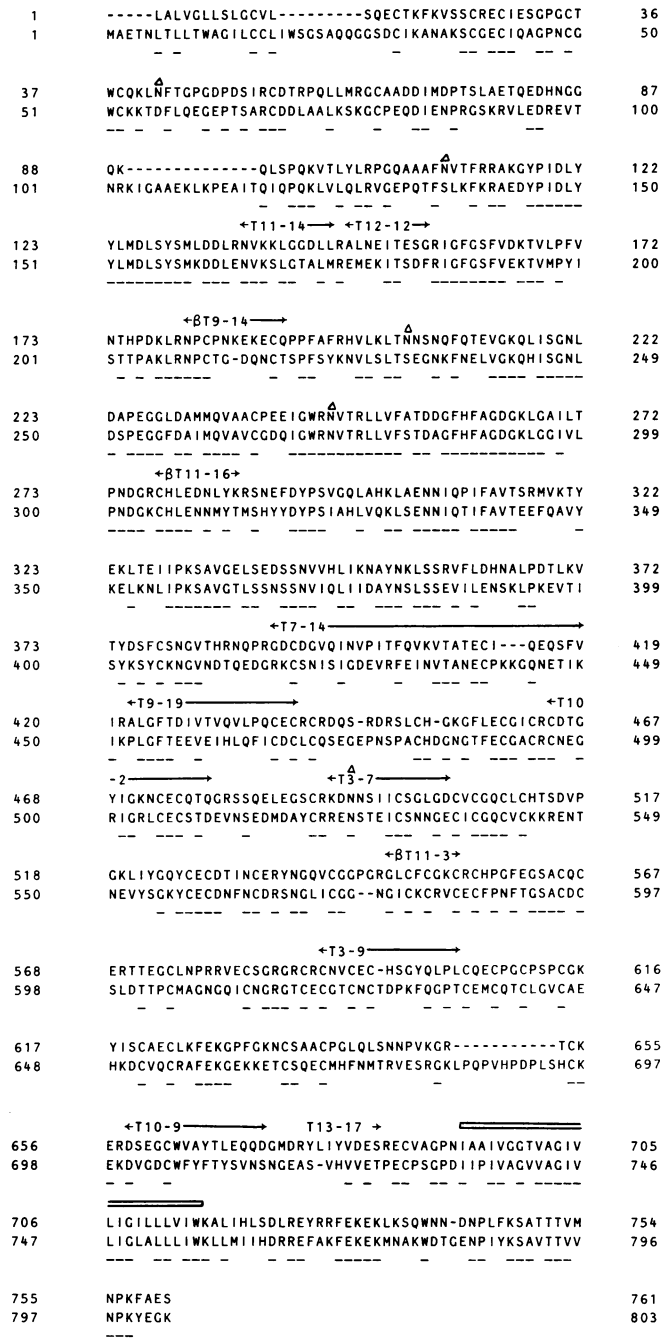

Fig. 3. The primary structure of the β-subunit of the cell surface adhesion glycoproteins. The derived amino acid sequence is shown. The position of the 12 tryptic peptides (<——>), the five potential N-linked glycosylation sites (△), and the putative transmembrane segment (═══) are marked. The primary structure of integrin (Tamkun *et al.*, 1986) is shown as the lower sequence. The alignment of the two sequences was made by the ALIGN program (Dayhoff *et al.*, 1983). Identical residues are indicated by (-) below the sequences.

β-subunit purified and reported here must be formally regarded as that of the LFA-1 antigen. It must be stressed, however, that the β-subunits of the three antigen complexes are likely to be identical, based on: (i) the extensive sharing of antigenic epitopes, e.g. that of monoclonal antibodies IB4 (Wright *et al.*, 1983), MHM23 (Hildreth *et al.*, 1983) and TSl/18 (Sanchez-Madrid *et al.*, 1983); and (ii) the invariable finding that patients defective in the β-subunit are deficient in all three antigens on their cell surfaces. The fact that H-52 does not bind to CR3 and p150,95 could be explained by the simple assertion that the epitope on the β-subunit is masked by the α-subunits of the CR3 and p150,95 complexes.

The cDNA clones J-9, X-1 and J-19 have insert sizes between 2.7 and 2.9 kb and are close to the size of the mRNA from the PMA-stimulated U-937 cells (3−3.5 kb). An open reading frame was found in the consensus sequence starting from the 5′ end to nucleotides number 2284−2286 where a termination codon TAG is found. The sequence contains five possible N-linked carbohydrate sites at positions 42, 108, 204, 246 and 493, in agreement with the findings of Dahms and Hart (1985) who demonstrated the presence of five glycosylated peptides from the β-subunit. The sequence also contains a hydrophobic segment from residues 693−715. It is therefore likely that the N-terminal portion of the protein is the extracellular domain and the C-terminal cytoplasmic with one transmembrane segment in between. The clones do not include the ATG signal for the initiation methionine residue. However, it is likely that a portion of the leader sequence is included in clone J-19. Since the N-terminal of the β-subunit is blocked (Springer *et al.*, 1985b), it is not possible to pinpoint the exact N-terminal of the mature protein but it is tempting to speculate that it might be the glutamine residue (position 15) after the putative leader sequence fragment. If that is the case, the β-subunit would contain 747 residues and would have a mol. wt of 82 542 daltons. Together with the carbohydrate at the N-linked glycosylation sites, estimated to have a mol. wt of 3000 daltons per site, the mol. wt of the β-subunit is estimated to be 97 542 daltons which is in reasonable agreement with the apparent mol. wt by SDS−PAGE. Our most N-terminal peptide, T11-14, marked the first definitive sequence at residue number 137 (Figure 3).

A most unusual feature of the primary structure is its high cysteine content. A total of 57 cysteine residues were identified with 42 found within a stretch of 256 residues (residues 437−692) N-terminal to the transmembrane sequence. Within this cysteine-rich region, there appear to be three repeating units arbitarily defined from residues 473−525, 526−564, and 565−603 to contain eight cysteine residues arranged in a pattern of C X C (——) C (——) C (——) C X C X X C X C (——) where each X stands for an amino acid and the (——) represents stretches of amino acids with length ranging from 4 to 14 residues.

The three repeating segments were subjected to statistical analysis by the ALIGN program (Dayhoff *et al.*, 1983; Staden, 1986). The pairwise comparison yielded alignment scores of 3.59, 5.45 and 4.29 SD units; indicating that the probability of a chance relationship is less than 2×10^{-4} (Dayhoff *et al.*, 1983).

The pattern of cysteine residues in the cysteine-rich region is very similar to that first observed in a subunit of a fibronectin binding protein (integrin) from chicken fibroblasts (Tamkun *et al.*, 1986). In fact, the two proteins show a high level of similarity. The two sequences are easily matched with a 47% overall identity. In a stretch of 278 amino acid residues from positions 90 to 367, identities are found at the level of 63%. However, a proposed possible phosphorylation site at a tyrosine residue in integrin (position 788) is not found in the aligned β-subunit. This observation is consistent with the failure to phosphorylate the β-subunit of cell-surface adhesion glycoproteins under various conditions (Malhotra, 1986).

The exact roles of the LFA-1 and p150,95 in their respective interaction between effector and target cells are not clear. However, CR3 has been postulated to participate in phagocytic events mediated by activated macrophages (Wright and Silverstein, 1982). Possibly, it serves as a communicative bridge bet-

ween iC3b on opsonised particles on the outside and the actin network on the inside, the perturbation of which by drugs such as cytochalasin B was known to inhibit the phagocytic capacity of the cells (Axline and Reaven, 1974). Thus, CR3 may be compared with the fibronectin receptor, which bridges the extracellular fibronection and the intracellular actin systems (Hynes, 1981). The analogy between the two receptors may extend to the specific recognition between the receptors and their extracellular ligands. It has been established that the type III domain of the fibronection receptor recognises a sequence of RGD on the fibronectin molecule (Perschbacher and Ruoslahti, 1984). It is therefore intriguing to note that the tripeptide RGD is also found in the primary structure of C3 (de Bruijn and Fey, 1985). Furthermore, a synthetic peptide of C3 covering the RGD region was shown to have affinity for CR3 (Wright *et al.*, 1987).

The high structural homology between the β-subunit and integrin clearly strengthens the conjecture that these proteins are related and belong to an extended family of cell-surface proteins involved in various forms of adhesion reactions.

Materials and methods

Purification of the β-subunit of the cell surface adhesion glycoproteins

The methods were essentially those of Williams and Barclay (1986). The H-52 monoclonal antibody (Hildreth and August, 1985) was coupled to Sepharose 4B CL at 8 mg IgG per ml of beads. The NP40 extract of 70 g of human spleen was passed through a 35 ml H-52 column. The material eluted at pH 11.5 was passed through a second H-52 column of 8 ml packed volume. The antigen was eluted at pH 2.2. All buffers used contain 1 mM iodoacetamide, 0.2 mM phenylmethanesulphonyl fluoride, 2 μM 1,10-phenathroline, 1 μM leupeptin, 1 μM pepstatin A and 3 mM sodium azide. Antigenic activities throughout the purification procedure were determined by inhibition of an indirect radioactive binding assay (Williams and Barclay, 1986). The purity of the antigen was assessed by SDS–PAGE (Laemmli, 1970).

Protein chemistry

The β-subunit obtained from affinity chromatography was succinylated, reduced and alkylated with iodo[2-^{3}H]acetic acid (Christie and Gagnon, 1982). Tryptic peptides were obtained and fractionated by gel filtration (Sephadex G-75) followed by reverse phase h.p.l.c. (Christie and Gagnon, 1982; Williams and Gagnon, 1982). N-terminal peptide sequencing was carried out in an Applied Biosystems Gas Phase Sequencer with PTH amino acid detected by h.p.l.c.

cDNA library

RNA was subjected to sucrose gradient ultracentrifugation and the 28S$^+$, 18S$^+$ (Malhotra, 1986) according to standard procedures (Maniatis *et al.*, 1982). The RNA was subjected to sucrose gradient ultracentrifugation and the 20S$^+$, 18S$^+$ and 5S$^-$ fractions were pooled and poly(A)$^+$ selected by passing through an oligo(dT) cellulose column. cDNA was made from 5 μg of the 28S$^+$, 18S$^+$ and 5S$^-$ poly(A)$^+$ RNA using a cDNA synthesis kit (Amersham International UK) according to the manufacturers procedures. The cDNA was blunt-end ligated to the plasmid vector PAT-X and transfected to *Escherichia coli* MC-1061. The library was amplified 50-fold before storage at −70°C. The complexity of the library is ~ 300 000. About 62.5% of the colonies contain sizeable inserts which have an average length of 1.5 ± 1.0 kb.

cDNA cloning and sequencing

Oligonucleotides were synthesised by the phosphodiester method (Gait *et al.*, 1980). Initial screening of the cDNA library was with the ^{32}P-labelled oligonucleotide probes. Subsequent re-screening of the library for further clones was with cDNA inserts obtained from the initial positive clones (Maniatis *et al.*, 1982). Sequencing of cDNA was by the M13 method (Sanger *et al.*, 1977; Biggin *et al.*, 1983).

Data handling and analysis

Sequence data were handled by the computer program of Staden (1986). Assessment of sequence similarities was made using the ALIGN program (Dayhoff *et al.*, 1983). Alignment scores were calculated using the Mutation Data Matrix (250 PAMS) with a bias of +6 and gap penalty of +6. 100 random runs were performed to establish the mean random scores for the sequences.

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These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00057.