A Novel Transiently Expressed, Integral Membrane Protein Linked to Cell Activation

MOLECULAR CLONING VIA THE RAPID DEGRADATION SIGNAL AUUUA

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A novel cDNA clone termed E16 which codes for an integral membrane protein of 241 amino acids with six transmembrane domains was isolated from peripheral blood lymphocytes. The cDNA clone is 4000 base pairs in length and exhibits an unusually long 3′-untranslated region of about 3000 nucleotides. Its expression at the mRNA level is closely linked to cellular activation and division. In all myeloid and lymphoid cells, as well as in primary lymphocytes from peripheral blood, E16 transcripts are rapidly induced and rapidly degraded after stimulation. This pattern of expression is unusual for an integral membrane protein and resembles more closely the kinetic seen for protooncogenes and lymphokines in the T cell system. Its isolation was made possible by a novel approach especially designed to selectively clone cDNAs which exhibit such an expression kinetic. It is based on a combination of the differential screening of a subtracted cDNA library and the subsequent hybridization of the resulting phages to a short oligonucleotide (5′-TAAATAAATAAATA-3′). This oligonucleotide is complementary to a trimer of the rapid degradation signal (AUUUA) which is present as a single or reiterated motif in the 3′-untranslated region of many short-lived transcripts.

The functional activation of resting cells, as well as the differentiation of immature progenitors into more mature cell types, requires coordinate up- and down-regulation of the expression of certain sets of genes in the course of time. In addition to the molecules directly involved in the process of replication, other proteins that are part of the signaling cascades leading to cell activation, like protooncogenes, growth factors, and their receptors, also belong to this group. The latter are particularly interesting because of their tumorogenic potential when overexpressed, mutated, or involved in autocrine-stimulated growth (see Refs. 1-4).

A tight regulation at several levels ensures the transient activation of these genes during the cell cycle and prevents their uncontrolled expression that may contribute to the tumorogenic event. One such level of regulation that is found especially in a subset classified as immediate early genes (5) is the rapid decay of their corresponding mRNAs (6, 7). Although we have limited insight into the complexity of this process, a pentameric signal sequence (AUUUA) in the 3′-end of their mRNAs seems to be crucial for the rapid turnover (8, 9). This sequence occurs as a single or multimeric motif which can even be reiterated at various distances between the coding region and the polyadenylation site. The identification of proteins binding to this sequence motif has further underlined the functional significance of this signal, which was originally described by Shaw and Kamen (10).

Differential hybridization of subtracted induced versus uninduced cDNA libraries is a powerful method to enrich and isolate genes that are specifically expressed at the induction stage and has been successfully applied in the past (11-14). We have up to several hundred clones. Phenotypes and properties of a given number of differentially expressed clones. Here we describe the isolation and the molecular characterization of a new cDNA clone out of 248 Δ cDNA phages which belongs to this early and transiently expressed subpopulation by its specific hybridization to an oligonucleotide complementary to a reiterated signal sequence. It codes for an integral membrane protein which, as judged from the mRNA data, probably belongs to the earliest changes at the membrane level after activation in all cell types.

EXPERIMENTAL PROCEDURES

With a few modifications, the cell work and the molecular biological methods for subcloning, RNA isolation, and Northern blot analysis, as well as the conventional and differential screening of cDNA libraries, are described by Gaugitsch et al. (12).

Hybridization with the 14-mer Oligonucleotide—Recombinant λgt10 phages, plaque-purified by three rounds of differential screening as recently described (12), were dotted on a lawn of Escherichia coli NMS14 host bacteria and grown at 37 °C until lysis occurred. Filter replicas were generated in duplicates on Hybond N nylon membranes (Amersham International, Amersham, UK) by plaque lifting for 45 and 90 s. Denaturation and neutralization were done according to the protocol of Amersham, and filters were baked afterward for about 2 h at 80 °C without vacuum. The filters were prehybridized for 4-6 h with constant shaking in 6 × SSC, 0.5% sodium dodecyl sulfate, 5 × Denhardt’s reagent, and 100 μg/ml salmon sperm DNA at 4 °C. For hybridization the same solution containing 0.1% EMTA and the oligonucleotide probe was used. Hybridization was done overnight at 4 °C with constant shaking. The filters were washed at 4°C times for 10 min in 10 × SSC, followed by a 5-min wash at room temperature.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M80244.

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in 10 × SSC. The filters were exposed to Kodak XAR films with intensifying screens at −70 °C for various periods of time. The positive and negative control plasmids were alkali-denatured using 200 mM NaOH for 10 min at 95 °C, neutralized with 0.33 M Tris, pH 7.5, and a final concentration adjusted to 5 × SSC. 1 μl of this solution containing different amounts of DNA as described under "Results" were then hand-doped on nylon membranes and baked as described above. Oligonucleotides were "end-labeled" with T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) in a 20-50 μl standard reaction as described (15). Usually, 50 ng of an 11/14-mer oligonucleotide were labeled using a 2-fold molar excess of \( \gamma^32P \)ATP (>5000 Ci/mmol, Amersham) over 5'-ends of oligonucleotide. Labeled oligonucleotides were purified using Nensorb columns according to the manufacturer's protocol (Du Pont, Vienna, Austria).

The peak eluate was dried in a Speed Vac concentrator and resuspended in ethanol. The labeled oligonucleotides were used as probes. Phage DNA were loaded onto an 8.0. The solution was then directly added to the hybridization mix.

**Subcloning and Sequencing—cDNA inserts were excised by EcoRI (Boehringer Mannheim) restriction digest out of the αgt10 recombinant phages and subcloned into the plasmid vector pBSM13+ (Stratagene, San Diego, CA) for further restriction mapping (15).**

The alignment of the several resulting EcoRI fragments/phage to each other was determined by additional SstI (Boehringer) restriction analysis and Southern blotting to subcloned EcoRI fragments as probes. The SstI fragments cloned into M13 mp18 (Boehringer Mannheim) were also used for establishing the sequence across EcoRI sites in the total insert using specific oligonucleotides as primers. Later sequencing of the EcoRI fragments was done either by double stranded sequencing using T3 and T7 primers or synthetic oligonucleotides as recently described (12). Additionally, subfragments directionally excised with restriction enzymes were further subcloned into M13 mp18/19 (Boehringer Mannheim) and sequenced with the help of the universal primer and specific oligonucleotides using a Pharmacia kit system (Pharmacia, Uppsala, Sweden). Sequencing was done in completion on both strands.

**Primer Extension—**Primer extension was done using a 17-mer oligonucleotide (TB40 sequence, 5′-GCCAGCACAATGTTCCC-3′; position in the clone, 233-249) and the preamplification kit system (from GIBCO/BRL, Berlin, Germany). The oligonucleotide was labeled with \( \gamma^32P \)ATP and purified as described above. 4 ng of the primer, together with 5 μg of total T cell mRNA, were heated to 70 °C for 10 min. The following extension reaction was done for 30 min at 37 °C. After 5 min at 95 °C and treatment for 5 min with RNase H as described by the manufacturer, the reaction was extracted with phenol, precipitated using glycogen carrier, and resuspended in formamide loading dye. The reaction was heated to 96 °C for 2 min and loaded onto an 8 M urea, 6% polyacrylamide gel. Gels were fixed in 10% acetic acid and dried before exposure.

**Human RNAs—**They were purchased from Clonetech (Clonetech, Palo Alto, CA) and used for a Northern blot analysis as recently described (12).

**RESULTS**

**Differential Screening—**We have recently described the isolation of 137 αgt10 Clones from a cDNA library of Jurkat T cells stimulated for 6 h with PMA/PHA by differential hybridization with induced versus uninduced probes. Generated from poly(A⁺)-selected endoplasmic reticulum mRNA, this pool of clones is specifically enriched for membrane and secreted molecules. Repeatedly, we rescreened representative aliquots of this cDNA library differentially to ensure the isolation of even low abundant clones and thereby enlarged our recently published pool size from 137 to 516 (12). Subtraction of the known lymphokines IL2, IL3, IL4, IL5, IL6, GM-CSF, and γ-IFN by hybridization to oligonucleotide or cDNA probes reduced this number to 248 so far uncharacterized phages.

![FIG. 1. A compilation of 3'-noncoding sequences of several human transitory genes containing the rapid degradation signal. At the top are six members of the cytokine/lymphokine family; below are four human proto-oncogenes: IL2 (26), IL5 (27), IL6 (28), IL8 (29), GM-CSF (30), tumor necrosis factor (TNF) (23), c-fos (31), c-sis (32), c-myc (33), c-myb (34). The pentameric signal sequence AUUUA is underlined/overlined. The regions with the most reiterations of the signal were chosen. All sequences are shown in 5'3' orientation from left to right.](image-url)

Hybridization with an Oligonucleotide Complementary to the Rapid Degradation Signal—Since we are primarily interested in the transiently expressed genes in cell activation, we exploited the possibility of defining members of this transient subpopulation by hybridization to short oligonucleotides complementary to multimers of the rapid degradation signal (RDS), AUUUA. As oligonucleotide probes, we used a 14-mer (5′-TTAAATAAAATATA-3′) designated TBOI and an 11-mer (5′-ATAAAATATA-3′) designated TBOII which contain three and two complete RDS sequences, respectively. The sequence analysis of 16 possible lymphokines, including IL1a (16), IL4 (17), IL5 (18), IL7 (19), G-CSF (20), α-IFN (21), γ-IFN (22), lymphotoxin (23), HILDA (24), pAT464 (25), and the ones given in Fig. 1, has shown a full match to the 14-mer (TBOI) in about 20% of the cases. Therefore, we reasoned that it should be possible to define new members with a similar degree of reiterations using this approach. To establish suitable conditions for hybridization, we used GM-CSF cDNA cloned into the plasmid vector pBSM13+ as a positive control over the background of plasmid vector pBSM13+ alone. Fig. 2A illustrates this system showing the sequences of the 3'-end of the GM-CSF gene covering the RDS motif and the 14-mer oligonucleotide TBOI. Fig. 2B shows an autoradiogram of a dot blot hybridization under two different conditions (see "Experimental Procedures" and the legend to Fig. 2). Because the signal strength in the cold room was about twice as strong as that at room temperature and did not significantly increase the background (compare Fig. 2B), all further experiments were performed at 4 °C. The 11-mer TBOII fails to give specific signals under these conditions, although the 14-mer TBOI specifically recognizes IL6 (13 matches/1 mismatch to TBOI) and IL2 (12 matches/2 mismatches to TBOI) under the conditions chosen (data not shown).

The plaque-purified 248 uncharacterized Δ cDNA phages were subjected to a Benton Davis screening with TBOI as a probe. Fig. 3 shows the hybridization of one filter replica in duplicates with about 100 phages. In total, 36 positives out of 248 plaques were obtained, reducing the number of phages by about 85%. Cross-hybridization analysis further reduced the number of unique sequences to about 16.2 Partial nucleotide sequence was generated by double stranded sequencing of each cloned insert and immediately compared to GenBank (version 63.0) and EMBL (version 22.0) data bases.
The sequences that have been analyzed in detail, one previously unknown clone was found which is unique under the 36 phages. Restriction analysis of this clone, termed E16, showed two EcoRI fragments of about 500 and 750 bp. The bigger one was used for establishing the primary sequence for the first computer comparison.

RNA Analysis with E16—Our interest with the newly isolated clone was primarily focused on its mRNA induction kinetics in order to check the validity of our approach. Therefore, Jurkat T cells were stimulated with PMA/PHA at various time points, and then the total RNA was prepared and subjected to a Northern blot analysis with the 750-bp EcoRI fragment from E16 as a probe (Fig. 4). The constitutive gene GAPDH (35) was used as a control. One band is detected shortly below the 28 S rRNA, indicating that the 1250-bp insert of our Jurkat E16 clone does not represent a full-length copy. The expression kinetics of this new gene clearly match the predicted pattern since it is induced about 10-fold after 6 h of induction (compare Fig. 4, left: Northern blot analysis, lanes 1 and 3), and its mRNA is rapidly degraded (compare Fig. 4, left: Northern blot analysis, lanes 3 and 4). This exactly fits the scheme of its isolation via differential library screening and selection by the oligonucleotide.

To clarify the tissue specificity and to see whether this gene is also induced in other cell types, we isolated the total RNA of nine different cell lines which were either left unstimulated or were stimulated with PMA/ionomycin for 6 h (see Fig. 5). Additionally, we tested adult (brain, lung, liver) and embryonal (brain, lung, liver, kidney of a 4-month-old fetus) human tissues using 1 μg of poly (A+) RNA per Northern blot analysis. While we were unable to see any hybridization in the human adult and embryonal tissues (the GAPDH control was easily detectable), all of the tested cell lines expressed the E16 gene, however, to different degrees (compare Fig. 5). Laser densitometry and normalization to the GAPDH control gene revealed an up-regulation after stimulation in all lymphoid and myeloid cell linesages, while Hacat (keratinocyte cell line), HeLa (cervix carcinoma cell line), MM06 (fibroblast cell line), and MM6 (monocyte progenitor cell line). For comparison, Jurkat RNA seen in Fig. 4, lanes 1 and 3 (0 h, 6 h induced with PMA/PHA) is shown on the left. The probe, as well as the control hybridization, were as described in Fig. 4.
versus stimulated PBL. Therefore, they were isolated, cultured, and activated as recently described (12), and the total RNA was subjected to a Northern blot analysis with the 750-bp EcoRI fragment as a probe (Fig. 6). In quiescent T cells, no expression of the E16 gene is detected, paralleling the situation in other human organs. mRNA induction is seen as no expression of the E16 gene is detected, paralleling the situation in other human organs. RNA was subjected to a Northern blot analysis with the 750-bp EcoRI fragment. From the initial hybridization, a match of 12/14 of the TBOI oligonucleotide (position 3714-3724). Additionally, a second AUUUA pentameric signal sequence is seen in position 3689-3693. To determine to which extent the 5′-end is contained in our cDNA clone or whether substantial parts are still missing because of an inaccuracy in size determination from the Northern blot analysis, a primer extension was performed. As a control, in vitro synthesized RNA via the T7 polymerase from our cDNA clone was run in parallel (Fig. 8). Lane 2 gives the 241 nucleotides (N) extension from oligonucleotide TB40 (see “Experimental Procedures”) at the control RNA while lane 3 shows an identical reaction performed with 5 μg of total RNA. This clearly indicates that only a few nucleotides are missing at the 5′-end of the cDNA clone which is probably due to the eukaryotic cap structure and the generation of the PBL cDNA library via the S1 nuclease after the second strand cDNA synthesis. This confirms the first ATG in position 311 which is in the context for a good translational initiation site (37) as the most likely start for the deduced E16 protein. The open reading frame of this protein is 241 amino acids long and stops at a TAG codon in position 1034–1036.

A New Integral Membrane Protein with Six Potential Transmembrane Domains—Computer-aided analysis indicates seven hydrophobic regions of about 14–25 amino acids in a predominantly α-helix conformation (amino acids: 8–29; 54–73; 104–123; 127–147; 162–183; 189–212; and 218–231) (see Fig. 9). The seventh hydrophobic stretch is 14 amino acids long and, therefore, is probably too short to span the membrane one more time; all others are between 20 and 25 amino acids in length. An NH2-terminal signal peptide is missing which suggests that the amino terminus is located in the cytoplasm (38). Reinforcing the likelihood of this orientation, short strings of positively charged amino acids which usually serve as anchors for transmembrane domains at the cytoplasmic site are seen after the fourth and sixth domain (LRHRKPELER; WKNKPKW; positive residues underlined). A very characteristic feature of proteins with multiple transmembrane domains is the position of several negatively charged residues in one of those strings, a fact best seen in the acetylcholine receptor (LRHRKPELER; negative residues underlined) (39). One possible N-linked glycosylation site is found in amino acid position 74–76. Also remarkable is the existence of a potential leucine zipper that is found from amino acid 83 to amino acid 111, where the second leucine is substituted by a negatively charged residue (Leu-83, Glu-90, Leu-97, Leu-104, Leu-111; also see “Discussion”). This structural motif is known to be involved in ligand binding and ion transport in several other membrane proteins (40, 41). No substantial homology was seen in computer-aided comparisons to already known proteins in the PIR (version 27.0) or Swiss Protein data bases (version 17.0).

**DISCUSSION**

In the present study, we describe the successful application of a novel strategy to clone genes that are transiently expressed after cell activation. This approach is based on the use of a short oligonucleotide which represents a complementary trimer to the rapid degradation signal AUUUA as a radioactive probe in hybridizations to a large number of Δ cDNA clones. These have been previously generated via subtractive cloning and differential hybridization of uninduced versus induced probes. By using the RDS-specific oligonucleotide, we finally selected 36 positively hybridizing phages out of 248, which represents a reduction of about 85%. This level of selectivity is not only the result of the oligonucleotide hybridization but also due to the facts that (i) already known lymphokine genes that are transiently expressed as well had been subtracted beforehand and (ii) the time point chosen for induction (6 h) and the use of endoplasmic reticulum mRNA for the preparation of the library had prevented the finding of nuclear oncogenes like c-fos and c-sis, which also have the RDS motif and belong to the transiently expressed gene pool. The validity of this approach is demonstrated by the isolation of the new E16 cDNA clone and one IL2 cDNA clone which obviously escaped our presubtraction. It should be mentioned, however, that in the meantime we have also isolated two mitochondrial genes which represent false positives in terms of the predicted kinetical pattern at the RNA level.6 It is known that mitochondrial genes are transcriptionally up-regulated after cell activation which explains why they are contained in the Δ cDNA phage pool. In addition, the two genes also have a reiterated (AUUUA), sequence consistent

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6. E. Prieschl, unpublished results.
A Novel Transiently Expressed Integral Membrane Protein

![Restriction map and nucleotide and amino acid sequences of E16.](image)

**FIG. 7.** Restriction map, and nucleotide and amino acid sequences of E16. At the top, a restriction map of the 3984-bp E16 cDNA clone is shown. The coding sequence is indicated (boxed). At the bottom, the nucleotide and amino acid sequence of the E16 cDNA clone in the 3-letter code are given. On the right, the nucleotide and amino acid positions are indicated. The AUUU signals which are outlined in the text are underlined. GenBank accession number, M80244.

with their selection by the RDS-specific oligonucleotide. However, as evidenced by Northern blot analysis (data not shown), their corresponding mRNA is not rapidly degraded, probably because the mitochondrion lacks the necessary machinery for rapid mRNA degradation.

While we have shown that the RDS-specific oligonucleotide probe is suitable for the isolation of transiently expressed genes, an attempt to directly isolate them from a cDNA library made of total mRNA failed. Numerous mRNAs contain a repetitive Alu element with homologous sequences of the kind (UAAAU)_n to (UAAAU)_13 at the 3'-integration site of this element at the coding strand. This generates an (AUUU)_m sequence in the double stranded cDNA clones which is picked up by our oligonucleotide. While RNAs with these elements are extremely abundant in the hnRNA and a considerable portion is still found in total mRNA from cells (and therefore in cDNA libraries from this source), this number is diminished in the cytoplasmic mRNA population, and they are nearly absent in the endoplasmic reticulum portion of i Δ cDNA clones representing induced genes. Therefore, the combination of differential screening plus oligonucleotide hybridization is highly selective and seems to be the best technique.

The new clone we have isolated via this technique represents a novel integral membrane protein with seven hydrophobic stretches as outlined by a Kyte-Doolittle, as well as a Hopp and Woods, hydrophobicity plot. Computer-aided analysis using the Mac Prot program PLOT A/KKD for predicting protein transmembrane domains indicated, however, a transmembrane character for only the first six amino-terminal stretches via the relaxed quadratic, as well as the more stringent linear, calculation. In a Chou-Fasman analysis, those stretches are found to be predominantly in an α-helix conformation, the commonly found secondary structure for membrane-spanning regions. The lack of a signal peptide, the only suitable cleavage site prediction in a von Heijne analysis (38) with a score of 30 in amino acid position 75/76, suggests an orientation of our protein with an intracellular amino and
behavior is remarkable. While it is absent in human adult (brain, lung, liver, PBL) and embryonal (brain, lung, liver, kidney) tissues to our detection limit, it was found in every cell line tested. This broad expression pattern in rapidly dividing cell lines, its absence in the human tissues that have a lower proliferative potential, and its rapid induction in PBL after activation suggest a direct functional involvement of the E16 protein in the process of cell division in general. In this respect, it resembles the transferrin receptor, which is also generally found on proliferating cells but which, on the contrary, does not belong to the transiently, i.e. rapidly degraded, gene set (7). The rapid onset of expression, as well as the rapid degradation at the RNA level, is unusual for an integral membrane protein like E16. Up-regulated receptors like those for IL2 and transferrin in PBL are first seen at the RNA level around 14 h after activation, and expression is still at quite high levels 48 h after stimulation (7). The pattern seen for E16, however, more closely resembles a kinetic seen for most cytokines in PBL (7, 36). Therefore, it probably belongs to the earliest changes in the membrane which are observed after activation. Future studies regarding the exact cellular localization and potential function of the E16 protein will be addressed with the help of monoclonal antibodies and should further elucidate its precise role in the activation process.

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Fig. 8. Primer extension on T cell RNA. A 17-mer oligonucleotide (TB40: sequence 5'-GCCAGCCAAATTGTC-3', position 233–249) was used as a primer. The primer alone is shown in lane 1. A control reaction performed on in vitro synthesized RNA via the T7 promoter from the E16 cDNA clone is given in lane 2. Lane 3 shows an identical reaction performed on 5 μg of total RNA. Lane S shows an unrelated sequencing reaction taken along as a size marker. On the right, the extended nucleotides (N) are given. The 3'-end of the primer is labeled O N. The control extension goes to 241 nucleotides (232 nucleotides from the clone + 9 nucleotides from the EcoRI site and the T7 promoter).

Fig. 9. Kyte-Doolittle hydrophobicity plot. The six transmembrane domains predicted by the Mac Prot PLOT A/KKD program are numbered 1-6 from the amino to carboxyl terminus. The carboxyl terminus. It is interesting to note that the only N-linked glycosylation site found overlaps this signal sequence cleavage site. However, in our predicted model for the E16 protein, this sequence would face the cytosol and, therefore, probably will not be used. So far, this structure of six transmembrane domains with intracellular amino and carboxyl termini is found in the case of "A-type" potassium channels. Other characteristics, however, like the (RXX), motif and the leucine zipper motif which follows it in the S4 region of these channels, are numbered by the Mac Prot PLOT A/KKD program.
A Novel Transiently Expressed Integral Membrane Protein

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