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Biochemistry

Identification of cDNA encoding an additional α subunit of a human GTP-binding protein: Expression of three α_i subtypes in human tissues and cell lines

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ABSTRACT **The guanine nucleotide-binding proteins (G proteins), which mediate hormonal regulation of many membrane functions, are composed of α, β, and γ subunits. We have cloned and characterized cDNA from a human T-cell library encoding a form of α_i that is different from the human α_i subtypes previously reported [Didsbury, J. R., Ho, Y.-S. & Snyderman, R. (1987) *FEBS Lett.* 211, 160–164 and Bray, P., Carter, A., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. & Nirenberg, M. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5115–5119]. α_i is the α subunit of a class of G proteins that inhibits adenylate cyclase and regulates other enzymes and ion channels. This cDNA encodes a polypeptide of 354 amino acids and is assigned to encode the α_{i-3} subtype of G proteins on the basis of its similarity to other α_i-like cDNAs and the presence of a predicted site for ADP ribosylation by pertussis toxin. We have determined the expression of mRNA for this and two other subtypes of human α_i (α_{i-1} and α_{i-2}) in a variety of human fetal tissues and in human cell lines. All three α_i subtypes were present in the tissues tested. However, analysis of individual cell types reveals specificity of α_{i-1} expression. mRNA for α_{i-1} is absent in T cells, B cells, and monocytes but is present in other cell lines. The finding of differential expression of α_{i-1} genes may permit characterization of distinct physiological roles for this α_i subunit. mRNA for α_{i-2} and α_{i-3} was found in all the primary and transformed cell lines tested. Thus, some cells contain all three α_i subtypes. This observation raises the question of how cells prevent cross talk among receptors that are coupled to effectors through such similar α proteins.**

The G proteins are a family of guanine nucleotide-binding membrane proteins that link extracellular signals to changes in cellular function. All known G proteins are heterotrimers composed of α, β, and γ subunits (reviewed in ref. 1). Functional specificity of G proteins is determined in part by the α subunits, which bind GTP, although the $\beta\gamma$ subunits also appear to be important regulatory elements (2, 3).

Recent advances in molecular cloning of cDNAs corresponding to α subunits have revealed that these proteins comprise a gene superfamily with multiple forms within categories of α subunits (α_T, α_s, and α_i). Two forms of the α_T subunit of transducin, the G protein that transduces light-initiated signals in the retina, have been identified (4–8); one form is specific to rods and the other is specific to cones. There are four forms of α_s (9, 10), the α subunit that mediates activation of adenylate cyclase and Mg^{2+} transport (reviewed in ref. 1). These forms are related to each other by alternate mRNA splicing (9, 10). Thus far, only one form of α_o, the 39-kDa major pertussis toxin substrate in brain (11, 12), has been identified (13–15). cDNAs termed α_i cDNAs have been isolated from rat (13), mouse (16), bovine (17, 18), and human (19, 20) libraries. These were called α_i either on the basis of identity in amino acid sequence with a known pertussis toxin substrate (the 41-kDa protein from brain) or on the basis of a potential site for pertussis toxin modification. Although the name α_i comes from an initial association with adenylate cyclase inhibition, G proteins of this class are now thought to be involved in arachidonic acid release, inositol phospholipid turnover, ion channel activity, secretion, and other cell functions (21, 22).

We now report the identification of a cDNA encoding a human α_i-like protein that is different from the human cDNA reported by Didsbury *et al.* (19) or Bray *et al.* (20). A comparison of the reported sequences shows that the α_i-like molecules may be grouped into several subtypes. The first type was isolated from a bovine brain library and was found to correspond exactly to the 41-kDa pertussis toxin substrate from brain (17, 18). This type of α_i has recently been found also in rat and in human and is called α_{i-1} (14, 20). The most commonly found sequence is that reported by Itoh *et al.* (13), which has been designated α_{i-2} by Jones and Reed (14). Forms of α_i essentially identical to this α_{i-2} were also found in a mouse monocyte library (16), a bovine pituitary library (17)‖, and a human monocyte library (19). For α_{i-2}, the difference among species was only 3–5 out of 355 amino acids. The α_{i-2} form differs from α_{i-1} by 43 out of 354 amino acids. We have recently shown that the genes for α_{i-1} and α_{i-2} are located on different human chromosomes (23).

The form of α_i reported here** differs from both α_{i-1} and α_{i-2} but is almost identical to rat α_{i-3} (14), as it differs only by 5 amino acids. The existence of multiple α_{i-1}-like cDNAs within a species and the conservation of amino acid sequences among species suggest that the α_{i-1}-like molecules may be specific in their functions. Comparative analysis of their amino acid sequences can provide clues to the structural basis for such specificity.

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Abbreviations: G protein, guanine nucleotide-binding protein; α_T, the α subunit of transducin, the G protein found in the retina; α_s and α_i, α subunits of G proteins that activate and inhibit adenylate cyclase, respectively; α_o, the α subunit of a G protein of unknown function.

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‖Our recent analysis indicates that the bovine α_h (17) is almost identical to the α_i subtypes isolated from rat (13), mouse (16), and human (19) libraries. Therefore, α_h should be classified as α_{i-2}.

**The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03238).

MATERIALS AND METHODS

Cloning and Sequencing of cDNA and Genomic DNA from a Third Human α Subtype. A cDNA library was generated in the phage vector λgt11 (24) from the T-cell line CCRF-HSB-2 according to the method of Gubler and Hoffman (25) and was kindly provided to us by Lloyd Klickstein (Massachusetts General Hospital, Boston). Approximately 400,000 plaques of an amplified library were screened by using the *Xba* I–*Sma* I fragment from the insert of a bovine α_i cDNA clone (17) or the *Eco*RI–*Eco*RI fragment of bovine $\alpha_{i\text{-}2}$ (α_h), as a DNA probe. The *Eco*RI inserts of selected and plaque-purified cDNA clones were subcloned into pBR322 or pBS M13+ (Stratagene, San Diego, CA). Nucleotide sequences of cDNA were determined by using the dideoxy chain termination method (26). Appropriate DNA fragments were generated by using restriction enzymes or BAL-31 (Bethesda Research Laboratories) and were subcloned into the M13mp18 and M13mp19 replicative forms. The conditions for BAL-31 reactions were described in detail elsewhere (27).

A human genomic DNA library in λEMBL3, prepared by E. M. Fritsch (Genetics Institute, Cambridge, MA), was screened with the complete cDNA for $\alpha_{i\text{-}3}$ (see below). A 16-kilobase (kb) DNA fragment was identified, which contained 600 base pairs (bp) of 5′ flanking sequence and the first exon of $\alpha_{i\text{-}3}$. The nucleotide sequence of the 5′ transcribed, but untranslated, region and of the first exon agree exactly with that obtained for $\alpha_{i\text{-}3}$ (see Fig. 1). The first exon encodes 40 amino acids (S.K., J.G.S., and E.J.N., unpublished results). The 600-bp fragment was excised as a *Sal* I fragment, purified by agarose gel electrophoresis, and used as a probe for analysis of genomic DNA and mRNA.

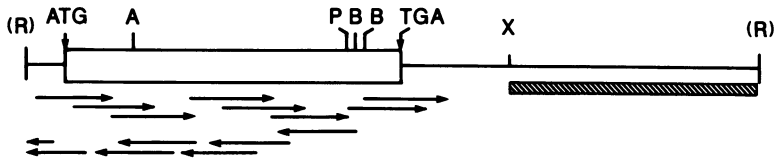
Identification of cDNA Clones for Human $\alpha_{i\text{-}1}$ and $\alpha_{i\text{-}2}$. Positive clones were identified in the T-cell library by using bovine α_h as a probe. The clone containing the longest insert was analyzed further. Sequence analysis of this clone showed absolute identity with the human cDNA reported by Didsbury *et al.* (19). Therefore, this cDNA was identified as encoding human $\alpha_{i\text{-}2}$. The radiolabeled fragment of bovine α_i (17) was used to screen a cDNA library prepared from human peripheral blood neutrophils kindly provided by Lloyd Klickstein. A cDNA clone with a 3.2-kb insert, which hybridized with bovine α_i but did not hybridize with either human $\alpha_{i\text{-}2}$ or human $\alpha_{i\text{-}3}$, was identified. The sequence of 477 5′ nucleotides of this clone was identical to the 3′ sequence of the human $\alpha_{i\text{-}1}$ cDNA previously reported (20). The remaining 2.7 kb correspond to the 3′ untranslated region of the longer $\alpha_{i\text{-}1}$ mRNA (see Fig. 3). An *Eco*RI–*Bam*HI fragment of this $\alpha_{i\text{-}1}$ cDNA [nucleotides 867–1344 in the published sequence (20)] was radiolabeled by nick-translation and used as a probe in these studies.

DNA and RNA Blot Analyses. Total genomic DNA was prepared from nuclei of human leukocytes as described by Bell *et al.* (28), digested with *Eco*RI, and subjected to Southern blot analysis. The filters were hybridized with appropriate probes at 42°C overnight as described (29). The final wash of the filters was at 65°C in 0.2× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0)/0.1% $NaDodSO_4$. Total cellular RNA was prepared by the guanidine isothiocyanate/cesium chloride method (30), separated by agarose gel electrophoresis, and transferred to nitrocellulose filters. The filters were hybridized at 42°C overnight with the appropriate probe and washed as above.

RESULTS

Characterization of cDNA and Genomic DNA for a Third Human α_i Subtype. Sixty positive clones were identified from the T-cell library, and 10 were plaque-purified. The cDNA

A



B

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-89  CGCCCAGCAATAGACGGTGCCTCAGCCTGCCGAGCCGCAGTTTCCGTGGTGTGAGTGAGTCCGGCCCGTGTCCCCTCTCCCGCCGCCGCCATGGGCTGC   9
                                                                                        MetGlyCys

 10  ACGTTGAGCGCCGAAGACAAGGCGGCAGTGGAGCGAAGCAAGATGATCGACCGCAACTTACGGGAGGACGGGGAAAAAGCGGCCAAAGAAGTGAAGCTG  108
     ThrLeuSerAlaGluAspLysAlaAlaValGluArgSerLysMetIleAspArgAsnLeuArgGluAspGlyGluLysAlaAlaLysGluValLysLeu

109  CTGCTACTCGGTGCTGGAGAATCTGGTAAAAGCACCATTGTGAAACAGATGAAAATCATTCATGAGGATGGCTATTCAGAGGATGAATGTAAACAATAT  207
     LeuLeuLeuGlyAlaGlyGluSerGlyLysSerThrIleValLysGlnMetLysIleIleHisGluAspGlyTyrSerGluAspGluCysLysGlnTyr

208  AAAGTAGTTGTCTACAGCAATACTATACAGTCCATCATTGCAATCATAAGAGCCATGGGACGGCTAAAGATTGACTTTGGGGAAGCTGCCAGGGCAGAT  306
     LysValValValTyrSerAsnThrIleGlnSerIleIleAlaIleIleArgAlaMetGlyArgLeuLysIleAspPheGlyGluAlaAlaArgAlaAsp

307  GATGCCCGGCAATTATTTGTTTTAGCTGGCAGTGCTGAAGAAGGAGTCATGACTCCAGAACTAGCAGGAGTGATTAAACGGTTATGGCGAGATGGTGGG  405
     AspAlaArgGlnLeuPheValLeuAlaGlySerAlaGluGluGlyValMetThrProGluLeuAlaGlyValIleLysArgLeuTrpArgAspGlyGly

406  GTACAAGCTTGCTTCAGCAGATCCAGGGAATATCAGCTCAATGATTCTGCTTCATATTATCTAAATGATCTGGATAGAATATCCCAGTCTAACTACATT  504
     ValGlnAlaCysPheSerArgSerArgGluTyrGlnLeuAsnAspSerAlaSerTyrTyrLeuAsnAspLeuAspArgIleSerGlnSerAsnTyrIle

505  CCAACTCAGCAAGATGTTCTTCGGACGAGAGTGAAGACCACAGGCATTGTAGAAACACATTTCACCTTCAAAGACCTATACTTCAAGATGTTTGATGTA  603
     ProThrGlnGlnAspValLeuArgThrArgValLysThrThrGlyIleValGluThrHisPheThrPheLysAspLeuTyrPheLysMetPheAspVal

604  GGTGGCCAAAGATCAGAACGAAAAAAGTGGATTCACTGTTTTGAGGGAGTGACAGCAATTATCTTCTGTGTGGCCCTCAGTGATTATGACCTTGTTCTG  702
     GlyGlyGlnArgSerGluArgLysLysTrpIleHisCysPheGluGlyValThrAlaIleIlePheCysValAlaLeuSerAspTyrAspLeuValLeu

703  GCTGAGGACGAGGAGATGAACCGAATGCATGAAAGCATGAAACTGTTTGACAGCATTTGTAATAACAAATGGTTTACAGAAACTTCAATCATTCTCTTC  801
     AlaGluAspGluGluMetAsnArgMetHisGluSerMetLysLeuPheAspSerIleCysAsnAsnLysTrpPheThrGluThrSerIleIleLeuPhe

802  CTTAACAAGAAAGACCTTTTTGAGGAAAAAATAAAGAGGAGTCCGTTAACTATCTGTTATCCAGAATACACAGGTTCCAATACATATGAAGAGGCAGCT  900
     LeuAsnLysLysAspLeuPheGluGluLysIleLysArgSerProLeuThrIleCysTyrProGluTyrThrGlySerAsnThrTyrGluGluAlaAla

901  GCCTATATTCAATGCCAGTTTGAAGATCTGAACAGAAGAAAAGATACCAAGGAGATCTATACTCACTTCACCTGTGCCACAGACACGAAGAATGTGCAG  999
     AlaTyrIleGlnCysGlnPheGluAspLeuAsnArgArgLysAspThrLysGluIleTyrThrHisPheThrCysAlaThrAspThrLysAsnValGln

1000 TTTGTTTTTGATGCTGTTACAGATGTCATCATTAAAAACAACTTAAAGGAATGTGGACTTTATTGAGAAGCATGGATGTTAGTGAAAGTTACTACAGTG 1098
     PheValPheAspAlaValThrAspValIleIleLysAsnAsnLeuLysGluCysGlyLeuTyrEnd
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FIG. 1. Structure and nucleotide sequence of the cDNA insert. (*A*) Restriction map and sequencing strategy. Restriction sites: A, *Acc* I; B, *Bgl* II; P, *Pvu* II; R, *Eco*RI; X, *Xba* I. ATG and TGA are start and stop codons, respectively. Horizontal arrows indicate directions and ranges of sequences read. The region indicated with a hatched box was used as the 3′ DNA probe in Fig. 2. (*B*) Nucleotide sequence and deduced amino acid sequence for the cDNA. Numbering is for the nucleotide sequence; the start codon is codon 1. The nucleotide sequence shown here includes the coding region and its immediate 5′ and 3′ flanking sequence.

clone with the longest insert, approximately 2.2 kb, was selected for further characterization and is described here. DNA fragments for sequence analysis were generated by using restriction sites available in the cDNA and the exonuclease BAL-31 (Fig. 1*A*). Fig. 1*B* shows the nucleotide sequence of the cDNA and the deduced amino acid sequence. The open reading frame contains 354 amino acids. Its calculated molecular mass, 41 kDa, agrees well with that of identified pertussis toxin substrates (1, 11). The amino acid sequence deduced from this cDNA easily aligns with the other α_i subtypes and α_o, and it is very similar to that reported for other α_i-like cDNAs but is different from both α_{i-1} and α_{i-2}. The difference from the former is 21 out of 354 amino acids, and the difference from the latter is 49 out of 354 amino acids. The difference from rat α_{i-3} is much smaller; only 5 out of 354 amino acids are different (14). Therefore, we consider this cDNA to encode human α_{i-3}.

Southern blots of human genomic DNA digested with *Eco*RI were probed with the ^{32}P-labeled DNA fragments of the human α_{i-3} cDNA and genomic DNA. When the whole 2.2-kb cDNA insert (Fig. 1) was used as a probe, six major bands appeared, which ranged in size from 3.3 to 15 kb (Fig. 2). A specific probe for this form of α_i was generated from a 600-bp DNA sequence isolated from the human genomic DNA, which includes the 5′ flanking region of the gene; the 3′ end of the fragment is located 8 bp upstream of the start codon. With this probe, only one band at 15 kb appeared, even after a long exposure under the same hybridization and washing conditions as those used for the total cDNA. Thus, the probe appears to be specific for this α_{i-3} gene. When the 3′ untranslated region of the cDNA (1-kb *Eco*RI–*Xba* I fragment; Fig. 1*A*) was used as a probe, three bands appeared. It is not yet clear if the appearance of such multiple bands results from the presence of intron(s) in the 3′ untranslated sequence and/or a homology in this region among closely related genes.

Expression of Three Forms of α_i in Human Tissues and Cells. Fig. 3 *B*, *D*, and *F* shows that all three forms of α_i are expressed in the human tissues examined. The three cDNA probes used each detect multiple specific messages. Under the hybridization conditions used, there is no crossreactivity of the probes. For α_{i-1}, we detected the 2.2-kb mRNA species previously reported (20) and an equally prominent 3.9-kb mRNA. Bray reported one major 2.2-kb band and three minor bands in brain but not in liver. Our probe biases the results in favor of the 3.9-kb message. The α_{i-2} probe detected a predominant mRNA of about 2.7 kb with minor bands at 3.4 kb and 1.7 kb, whereas the α_{i-3} probe detected a predominant mRNA of about 2.8 kb.

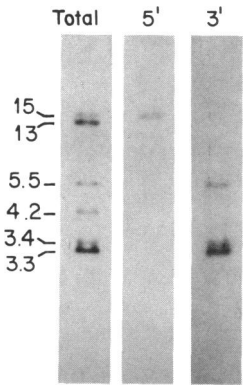

FIG. 2. Southern blot analysis of human genomic DNA. Human genomic DNA was digested with *Eco*RI. Blots were probed with the full length cDNA insert (Total; see Fig. 1), the 5′ flanking sequence (5′; see text), and the 3′ untranslated, but transcribed, region (3′; see Fig. 1). The sizes (in kb) of DNA fragments were estimated from the bacteriophage λ DNA digested with *Hin*dIII, which was run on the same gel.

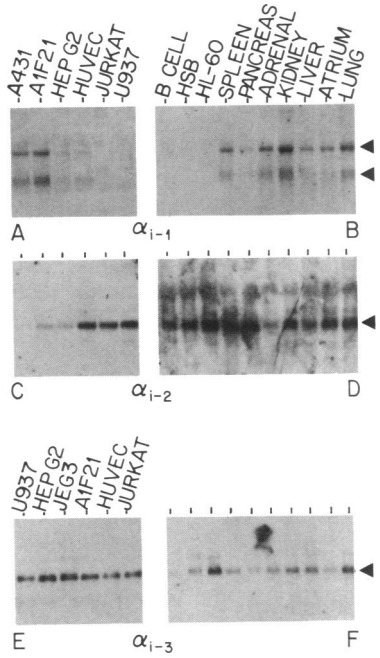

FIG. 3. Analysis of mRNA from human fetal tissues and human cell lines. Total cellular RNA was extracted from human fetal tissues and cell lines. Blots were hybridized with probes for α_{i-1}, α_{i-2}, and α_{i-3}. (*A* and *B*) Blots were hybridized with the *Eco*RI–*Bam*HI fragment containing nucleotides 867–1344 of the published α_{i-1} sequence and an additional 3′ untranslated region corresponding to the larger mRNA. The arrowheads mark the positions of the 3.9- and 2.2-kb bands. (*C* and *D*) Blots were hybridized with the full-length *Eco*RI insert of human α_{i-2}. The arrowhead marks the predominant 2.7-kb band. (*E*) The blot was hybridized with a 600-bp fragment derived from the 5′ flanking region of the α_{i-3} gene as described in the text. (*F*) The blot was hybridized with the *Hin*dIII–*Hin*dIII fragment of α_{i-3} cDNA. The arrowhead marks the predominant 2.8-kb band. The blots were washed at 65°C in 0.2× SSC/0.1% NaDodSO$_4$. The cell lines are A431 (epithelial carcinoma), A1F21 (human dermal fibroblast), HepG-2 (hepatoma), HUVEC (human endothelial cells), Jurkat (T cell), U937 (monocyte), B cell (Epstein–Barr virus transformed peripheral blood B cells), HSB (T cell), and HL-60 (monocyte).

The observation that mRNA for all three α_i subtypes was expressed in all the fetal human tissues examined led us to inquire whether all the subtypes were expressed in a single cell or whether there was differential expression in single cell types. The results are also shown in Fig. 3. Fig. 3 *A* and *C* show the results of probing the same nitrocellulose filter with cDNA probes specific for α_{i-1} and α_{i-2}. It is clear that the relative abundance of the mRNA for these two subtypes is variable. As shown in Fig. 3 *C* and *D*, α_{i-2} is found in all the cell types, but expression of α_{i-1} seems to be more cell specific since T and B lymphocytes and monocytes contain no α_{i-1} mRNA (Fig. 3 *A* and *B*). In addition to the cell lines shown, interleukin 2-dependent T-cell clones and a B-cell line (Ly65) were also negative (data not shown).†† Fig. 3 *E* and *F* show expression of α_{i-3} in nine human cell lines. In addition, A431 cells, included in Fig. 3 *A* and *C* but not *E*, were found in separate experiments to express α_{i-3} mRNA (data not shown).

DISCUSSION

The α_i subunits of G proteins are involved in a variety of cell responses, such as regulation of adenylate cyclase activity, phosphoinositol metabolism, or ion channel activity. The

††We do not know what cell is the source of the α_{i-1} cDNA we isolated from a peripheral neutrophil library.

discovery of multiple α_i subtypes raises a number of important questions. Is a subtype specific for a particular effector function or for a particular receptor? What part of the α_i structure is likely to determine that specificity? The comparative analysis of amino acid sequences among α subunits may provide clues to answers. Fig. 4*A* shows a comparison of the amino acid sequences of the α_i subtypes (i.e., human α_{i-1} vs. human α_{i-3} and human α_{i-2} vs. human α_{i-1}), whereas Fig. 4*B* shows a comparison of human α_{i-3} with rat α_o (14). The solid bars in Fig. 4 indicate the four highly conserved regions of the α_i and α_o proteins that are similar to sequences in the *ras*-encoded proteins and the bacterial elongation factor EF-Tu (31–33). In EF-Tu, these regions make up the GDP-binding pocket and, therefore, are presumed to form the GTP-binding site in the α proteins as well. These four regions are identical in all the α_i subtypes and α_o. However, they are subtly different from equivalent regions in α_s (10, 13, 34).

It is also apparent from Fig. 4 that the sequence differences among the α_i subtypes and α_o are not uniformly distributed along the polypeptide. The major region of difference is located between amino acid residues 80 and 130. Within α_i subtypes, the variable region is conserved across species, suggesting that a specific function of the α subunit may be determined in part by this region. In the *ras*-encoded protein, the sequence between the A and C regions of the GTP-binding site (Fig. 4) is necessary for transformation and, therefore, may interact with the cellular target for *ras* (35). By analogy, this region in α proteins has been proposed to be the effector domain (36). We have recently shown that in α_o alkylation of Cys-108 within the potential effector domain can block ADP ribosylation of residue 347 by pertussis toxin (37).

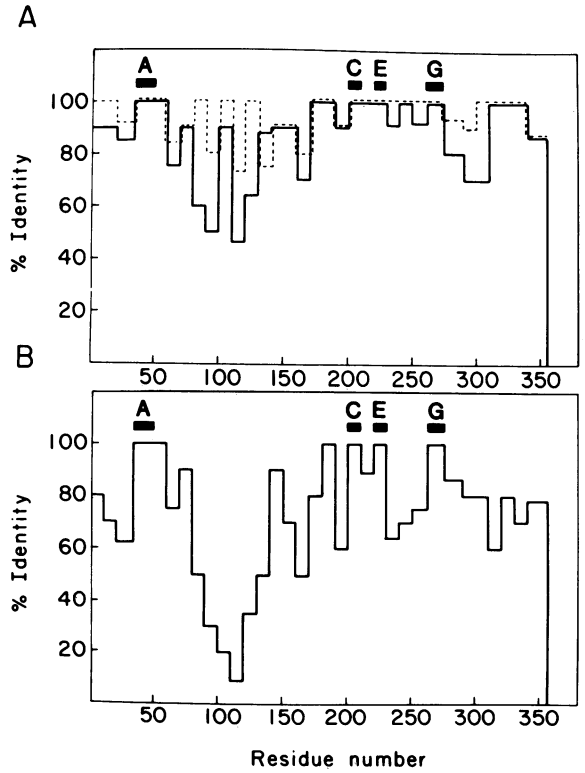

FIG. 4. Comparison of amino acid sequences among α_i subtypes and α_o. (*A*) - - -, $\alpha_{i-1}/\alpha_{i-3}$; ——, $\alpha_{i-2}/\alpha_{i-3}$. (*B*) α_o/α_{i-3}. The number of identical amino acid residues in a given segment was expressed as a percentage and was plotted as a function of the amino acid residue number of the respective proteins. The length of the segment (11 ± 3 residues) was determined to maximize the identity. The human α_{i-2} has 355 amino acids, one more than all the others. Therefore, a gap was introduced at position 123. The solid bars (A, C, E, and G) indicate highly conserved regions of the α_i and α_o proteins.

This observation demonstrates that structural changes within the variable region can profoundly affect the function of the α subunit.

The identification of multiple α_i-like cDNAs provides the basis for beginning to assign the different functions ascribed to α_i-like G proteins to particular members of the family. Comparative analysis of their sequences suggests specific regions to be targeted for structural analysis.

Another question raised by the existence of multiple α_i subtypes is whether these subtypes are segregated into different cells, as is the case with the two transducin α subunits (8) or whether they are found in single cells. Our results show that several human cell lines contain all three α subunits. Therefore, it is likely that the cell will need internal mechanisms to prevent cross talk among receptors that are coupled to their effectors through such extremely similar G proteins.

However, all three subtypes are not invariably coexpressed. None of the T, B, and promyelocytic cell lines tested contained detectable α_{i-1} mRNA. The functional consequences of this absence are not yet known, but differential expression of α_i genes may permit characterization of distinct physiological roles for the G proteins.

Note. While this paper was under review, Didsbury and Snyderman (38) published the sequence of a human cDNA identical to the one reported here.

We thank Dr. Randall R. Reed for providing the nucleotide sequences of the rat α_i, α_o, and α_s before publication. We are also grateful for the generous assistance of Dr. F. Bieber, Dr. Christine Seidman, Dr. Lloyd Klickstein, and Ms. Karen Klein in various aspects of the work. This work was supported by a Leukemia Society Postdoctoral Fellowship (D.B.B.), a Pfizer Pharmaceutical Postdoctoral Fellowship (K.D.B.), a National Institutes of Health Postdoctoral Fellowship (C.T.), Physician Scientist Award HL01835 (R.L.), National Institutes of Health Grants AI 18436 and AI 19148 (J.G.S.), and National Institutes of Health Grant GM 36259 (E.J.N).

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