the number of cellular events that could be responsible for the expression of the potentiation effect. Specifically, it would seem that the differential contribution of the two receptor types to LTP is incompatible with the idea that the potentiation effect results from an increase in transmitter release by a fixed population of terminals (*12*), since this would presumably affect both types of receptors. So it is noteworthy that NMDA-mediated responses exhibit robust paired-pulse facilitation, a transient presynaptic form of plasticity; moreover, binding studies suggest that NMDA receptors are at least as numerous as quisqualate receptors in area CA1 of hippocampus (*13*) and in postsynaptic densities (*14*).

On the other hand, several postsynaptic mechanisms could account for the selective changes produced by LTP. A first possibility would be a modification of the biophysical properties of the quisqualate, but not NMDA, receptor-ionophore complex, such as changes in the major conductance state of the channel (*15*). Other explanations are suggested by electron microscopic experiments that show that LTP is correlated with a change in the morphology of dendritic spines and, perhaps related to this, an increase in the number of synaptic profiles located on dendritic shafts and of spines with very short necks (*16*). Formation of new synapses lacking NMDA receptors (*17*) would account for the pattern of results described here, while changes in the shape (and thus biophysics) of existing spines might also produce the observed effects by differentially affecting the fast rising (non–NMDA receptor–mediated) versus more slowly developing (NMDA receptor–mediated) synaptic responses. Some models of dendritic spines suggest that changes in spine neck parameters could have effects of this type (*18*).

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Molecular Cloning of Two Types of GAP Complementary DNA from Human Placenta

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The *ras* p21 GTPase-activating protein (GAP) was purified from human placental tissue. Internal amino acid sequence was obtained from this 120,000-dalton protein and, by means of this sequence, two types of complementary DNA clones were isolated and characterized. One type encoded GAP with a predicted molecular mass of 116,000 daltons and 96% identity with bovine GAP. The messenger RNA of this GAP was detected in human lung, brain, liver, leukocytes, and placenta. The second type appeared to be generated by a differential splicing mechanism and encoded a novel form of GAP with a predicted molecular mass of 100,400 daltons. This protein lacks the hydrophobic amino terminus characteristic of the larger species, but retains GAP activity. The messenger RNA of this type was abundantly expressed in placenta and in several human cell lines, but not in adult tissues.

THE *ras* P21 GTPASE-ACTIVATING protein (GAP) was first identified as a cytoplasmic factor from *Xenopus* oocytes that greatly stimulated GTPase activity of normal N-*ras* p21 without affecting oncogenic mutants (*1*). Presence of this factor explained how normal p21 proteins could exist in their inactive, GDP-bound states in vivo, while oncogenic mutants remain GTP-bound and thus fully active. Subsequently, it was shown that extracts from many, if not all, types of higher eukaryotic cells contain GAP activity, and that GAP interacts with *ras* p21 proteins at a site that is essential for *ras* action (*2, 3*). This site, the putative effector binding site, corresponds to amino acids 32 to 40 of mammalian p21 proteins; deletions or certain point mutations in this region prevent transformation of cells by v–H-*ras* and prevent activation of *Saccharomyces cerevisiae* adenylate cyclase in vitro (*4*). Since GAP interacts at this site, it is likely that GAP itself is an effector of *ras* action, and that elucidation of other biochemical properties of GAP may shed light on the role of *ras* oncogenes in cellular transformation.

Recently, Gibbs and co-workers purified GAP from bovine brain and confirmed that this purified protein, which has an apparent size of 125,000 daltons, was able to stimulate GTPase activity of normal H-*ras* p21 without affecting GTPase activity of position 12 mutants (*5*). By means of the sequence obtained from this purified protein, a cDNA clone encoding bovine GAP was obtained (*6*). Furthermore, it was shown that oncogenic forms of *ras* p21 in their GTP-bound forms bind to GAP; this observation strongly supports the suggestion that GAP is an effector of *ras* action. In this paper, we describe the purification of GAP from human placenta and the molecular cloning of cDNA encoding this protein. In addition, we describe a second form of GAP cDNA that could encode GAP with an altered NH_2-terminus; we also demonstrate

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tissue-specific expression of these two forms.

Human GAP was purified to about 85% homogeneity from freshly processed placenta. Details of GAP purification will be published elsewhere (*7*). Briefly, proteins were precipitated from 100,000*g* cytosolic extracts with 40% ammonium sulfate and were allowed to adsorb to an S-Sepharose cation exchange column. Eluted fractions containing GAP activity were then applied to a DEAE HPLC anion exchange column, followed by a phenyl HPLC column. At this point in the purification, a protein band of apparent M_r 120,000 was observed to copurify with GAP activity. One hundred fifty micrograms of this protein were obtained from 300 g wet weight of human placenta. The 120,000-dalton protein was eluted from an SDS-polyacrylamide gel and digested with lysyl endopeptidase. NH_2-terminal sequences of the isolated peptides were determined. Oligonucleotides corresponding to the most probable DNA sequence of one of these (*8*) were synthesized and used to screen a lambda gt11 cDNA library from human placental mRNA. One strongly hybridizing plaque was picked, subcloned into M13mp18 and sequenced. This clone (referred to as GAP6) contained a DNA sequence corresponding to amino acid sequence obtained from the purified 120,000-dalton protein and appeared to be a partial GAP cDNA.

The cDNA clones were obtained by rescreening the library under stringent conditions with an oligonucleotide derived from GAP6 sequence. DNA inserts from 163 positive plaques were analyzed by the polymerase chain reaction (PCR) (*9*), in which an oligonucleotide corresponding to the 5′ end of GAP6 in the antisense direction was used as one amplifying primer, and an oligonucleotide from the lambda gt11 vector was used as the second amplifying primer (*10*). With this technique, it was possible to estimate the amount of sequence 5′ to known GAP6 sequence in each plaque. Plaques with the most new 5′ sequence were further analyzed by direct sequencing of PCR-amplified DNA (*11*); this procedure facilitated rapid analysis of these plaques and identification of seven clones with larger inserts for further analysis. These inserts were subcloned into M13mp18 for DNA sequencing.

Of the seven clones analyzed, five appeared to represent different length fragments of the same type of transcript. The longest of these, clone 101, would encode a protein of 1047 amino acids, with a predicted molecular size of 116,000 daltons (Fig. 1). The amino acid sequence predicted from clone 101 cDNA is 96% identical to that of bovine GAP (*6*). Most of the differences lie within the first 150 amino acids, which is the region that is not necessary for GTP activation (*6*) and contains the hydrophobic NH_2-terminus. Specifically, 31 out of 40 single amino acid substitutions between bovine and human GAP occur in this region, and most of these substitutions (20/31) are conservative. Human GAP contains a repeat of the sequence Ala-Gly-Val-Ala-Gly within this region; this sequence is only present once in bovine GAP.

Two regions with striking homology between bovine GAP and cytoplasmic tyrosine kinases, phospholipase C-148, and the *crk* oncogene have been identified previously (*6*); these regions of homology, referred to

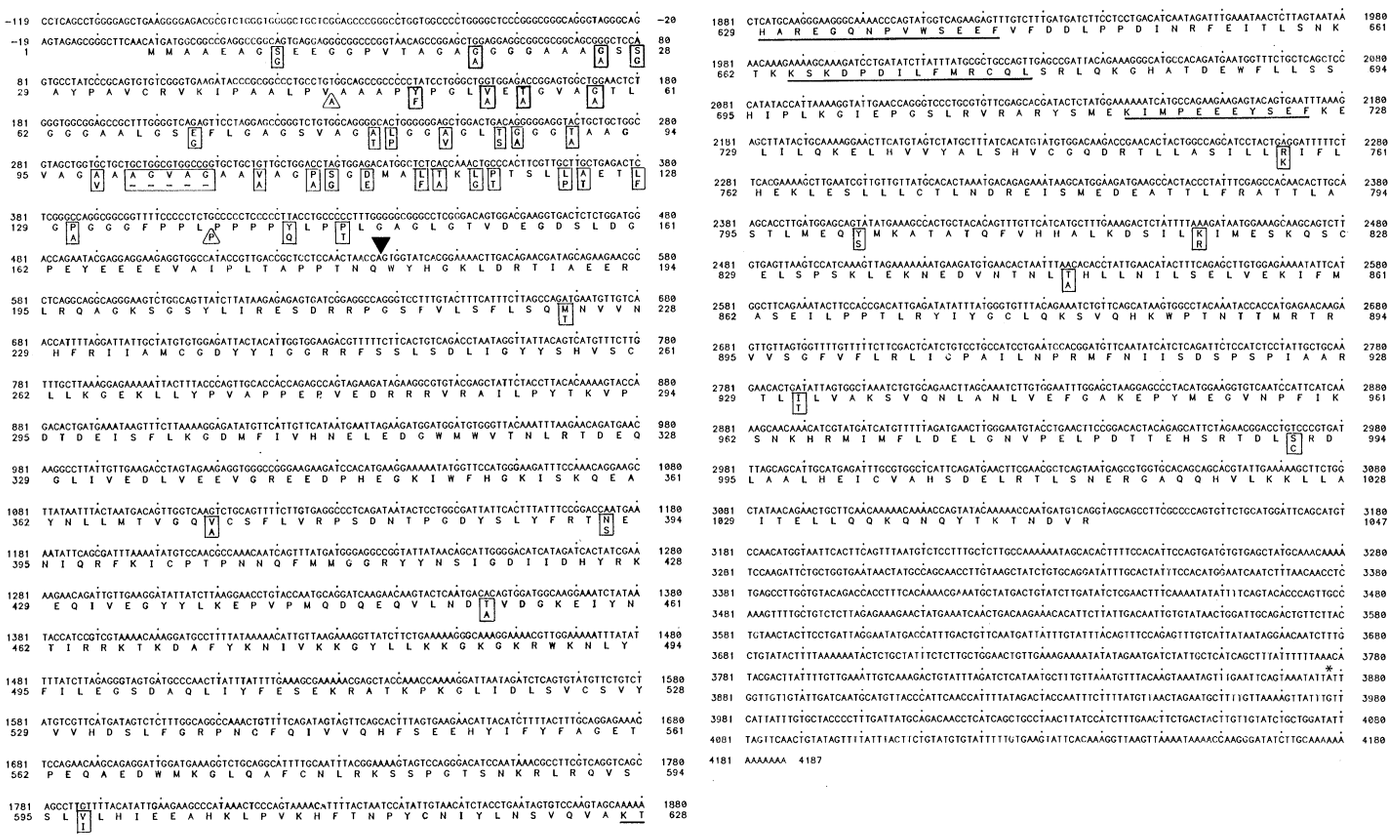

Fig. 1. Sequence of human GAP (clone 101). Sequences corresponding to peptides derived from proteolytic digestion of purified GAP are underlined. The position of the additional 65 bp found in clone 16 is indicated by a filled triangle. The sequence of this 65-bp insert is:

GTAAGT*TAA*GACTGCTGTTCAGGAATTTGGGAAGCTGGCTCCAGAAAAGAAGTGGAA*ATG*AAGGG

The termination and initiation codons in frame with 3′ GAP coding sequences are in italics. The 5′ end of clone 16 corresponds to nucleotide 496 of clone 101. Codons at which bovine GAP differs from human GAP are indicated by boxes. Amino acids present in bovine GAP with no equivalent in human GAP are in triangles. An asterisk indicates the position of poly(A) addition in clone 16. Single letter amino acid code: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

as SH2 regions (*12*), are identical in human and bovine GAP. Regions of bovine GAP that have statistically significant homology with *S. cerevisiae* adenylate cyclase were also reported (*6*); within the regions of homology reported, human GAP differs from bovine GAP by three amino acids, making the homology with adenylate cyclase is less significant.

Two other cDNA clones of a different type were also identified. These contained an additional 65 bp inserted between nucleotides corresponding to 537 and 538 of clone 101. One of these clones (clone 16) also differed from clone 101 in its site of polyadenylation (Fig. 1). Northern analysis of polyadenylated [poly(A)$^+$] RNA from human placenta, lung, liver, brain, and leukocytes was performed with an oligonucleotide expected to be specific for mRNA of the clone 101 type. This oligonucleotide spans the position of the 65-bp insert and should not hybridize efficiently to mRNA species containing this insert under stringent conditions. This oligonucleotide hybridized to a mRNA species of between 4.2 and 4.6 kb (Fig. 2); the actual size of GAP mRNA varied among the tissues tested, possibly as a result of alternative polyadenylation sites. Highest levels of expression were seen in placenta and brain. The filter was then stripped and reprobed with an oligonucleotide specific for the 65-bp insert. This species was also expressed strongly in placenta, but was not detected in other tissues (Fig. 2). The size of this species in placenta was approximately 3.8 kb. The abundance of this GAP mRNA in placenta suggests that this species is not simply the result of aberrant splicing. Analysis of the sequence of the insert shows that it is not unspliced intron, since no 3′ acceptor site is present (Fig. 1). The 5′ end of the insert represents a typical splice donor sequence (*13*) that was apparently not utilized in the generation of clone 16 type mRNA.

Preliminary data indicate that mRNA encoding this novel form of GAP is expressed in IMR-90 and MRC-5 cells (human diploid fibroblasts derived from fetal lung) and Namalwa cells (Burkitt's lymphoma) (*14*), as well as in placenta (Fig. 2). Since it does not appear to be expressed at detectable levels in several adult tissues, the possibility exists that the form of GAP encoded by this mRNA is associated specifically with proliferating cells. Of particular interest is the prediction that GAP encoded by mRNA of this type would initiate at a methionine within the 65-bp insert. This is the first ATG that appears in clone 16 cDNA, and it is preceded by a termination codon in the same reading frame (legend to Fig. 1). Clone 16 mRNA appears to be at least 150 bp shorter than clone 101–type mRNA at the 5′ end and may not contain the initiating codon of the latter type. If indeed the initiation occurs at the ATG within the 65-bp insert, the predicted protein would lack the hydrophobic NH_2-terminus characteristic of 120,000-dalton GAP and would have a molecular mass of 100,407 (870 amino acids). It seems likely that the absence of a hydrophobic NH_2-terminus would result in different interactions with other cellular components, particularly membranes. However, both forms of GAP, expressed in *Escherichia coli* or in insect cells, interact similarly with p21.GTP, since they are both capable of stimulating p21 GTPase activity (*15*).

In summary, we have purified GAP and have obtained internal amino acid sequence from this protein. Two classes of cDNA have been cloned using this amino acid sequence information. One class is predicted to encode a protein with molecular weight very similar to purified GAP and represents the human equivalent of bovine GAP cDNA (*6*). The other has a small insertion that appears to be the result of differential splicing; this would encode a significantly smaller protein with a different NH_2 terminal sequence. Both types of cDNAs produce protein with GAP activity. The availability of these clones should help elucidate the biochemical and biological function of GAP and the role it plays in *ras*-mediated cellular transformation and growth control.

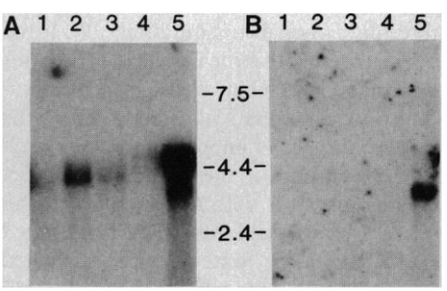

Fig. 2. Northern blot analysis of human RNA. Polyadenylated total cellular RNA (5 μg) was subjected to electrophoresis through a 1% agarose gel containing 2.2*M* formaldehyde as described by Maniatis *et al.* (*8*). The gel was blotted to Zeta-probe (Bio-Rad) and, in (**A**), probed with GM98, an antisense oligonucleotide which is clone 101–specific. The oligonucleotide (5′-CCGTGATACCACTGGTTAGT-3′) was phosphorylated with ^{32}P-ATP. The blot was hybridized in 20% formamide, 5× SSC, 5× Denhardt's solution, 10 m*M* $NaPO_4$, *p*H 7.0, 1% SDS, and yeast RNA (100 μg/ml) overnight at 42°C and washed in 2× SSC, 0.1% SDS at 42°C. After autoradiography for 7 days, the filter was stripped by washing in 0.1× SSC, 0.1% SDS at 90°C for 10 min, in (**B**), and reprobed with GW51 (5′-CTTCATTTCCACTTCTTTTCTGGAGCCAGCCTTCCC-3′), an antisense oligonucleotide which is specfic for insert sequence found in clone 16. The hybridization conditions were the same as above except the formamide concentration was 30%. Exposure time was 2 days. Lane 1, leukocyte mRNA; lane 2, brain mRNA; lane 3, lung mRNA; lane 4, liver mRNA; and lane 5, placental mRNA.

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47 (1988)] to ATT by site-directed mutagenesis, to generate pVL941 (V. A. Luckow and M. Summers, in preparation). A polylinker was inserted into the Bam HI site 30 bp 3′ to this ATT to generate pAcC9. Transfer vectors containing clone 101 or clone 16 GAP cDNA were transfected into Sf9 cells and recombinant virus prepared as in Luckow and Summers (1988). Four days after infection, cells were pelleted and extracts made with 0.5% NP-40, 10 m*M* tris-HCl (*p*H 8.0), 150 m*M* NaCl. Extracts were diluted into GAP assay buffer as above. These extracts could be diluted more than 10,000-fold without loss of GAP activity.

16. We thank J. Noble for help with sequencing and subcloning; E. O'Rourke and R. Clark for expression of GAP in insect cells; D. Spasic, L. Goda, and C. Levenson for oligonucleotides; H. Adari for helpful discussions; C. Jones and J. Murphy for typing the manuscript; and S. Nielson for graphics.

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Repair of the Secretion Defect in the Z Form of α1-Antitrypsin by Addition of a Second Mutation

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Homozygous inheritance of the Z-type mutant form of the α1-antitrypsin (α1AT) gene results in the most common form of α1AT deficiency, a human hereditary disease associated with a high risk for the development of emphysema and an increased incidence of neonatal hepatitis. The α1AT-synthesizing cells of individuals with the Z gene have normal α1AT messenger RNA levels, but α1AT secretion is markedly reduced secondary to accumulation of newly synthesized α1AT in the rough endoplasmic reticulum. Crystallographic analysis of α1AT predicts that in normal α1AT, a negatively charged Glu^{342} is adjacent to positively charged Lys^{290}. Thus the $Glu^{342} \rightarrow Lys^{342}$ Z mutation causes the loss of a normal salt bridge, resulting in the intracellular aggregation of the Z molecule. The prediction was made that a second mutation in the α1AT gene that changed the positively charged Lys^{290} to a negatively charged Glu^{290} would correct the secretion defect. When the second mutation was added to the Z-type complementary DNA, the resulting gene directed the synthesis and secretion of amounts of α1AT similar to that directed by the normal α1AT complementary DNA in an in vitro eukaryotic expression system. This suggests the possibility that a human hereditary disease can be corrected by inserting an additional mutation in the same gene.

THE SERUM GLYCOPROTEIN α1-ANTITRYPSIN (α1AT) is produced by hepatocytes and mononuclear phagocytes and is a major inhibitor of the powerful serine protease, neutrophil elastase (*1–4*). The α1AT gene is a 12-kb single-copy gene on chromosome 14 (*5*). The gene is pleomorphic, with 75 variants known (*2*). Between 1 and 2% of the α1AT alleles carried by Caucasians of northern European descent contain the "Z" type α1AT gene, an α1AT variant associated with reduced α1AT serum levels (*1, 2*). When the Z gene is inherited in a homozygous fashion, serum α1AT levels are 3.0 to 5.8 μ*M*, as compared to the normal 20 to 48 μ*M* (*1, 2*). This decrease results in an insufficient amount of α1AT to protect the lower respiratory tract from the ravages of neutrophil elastase, and the individual is at high risk for the eventual development of emphysema (*6*).

The Z α1AT gene differs from the normal M1(Ala^{213}) gene by a single base G → A change in the codon GAG of Glu^{342} that changes the residue to Lys (*7, 8*). The homozygous Z "deficiency" state results from post-translational events occurring in the rough endoplasmic reticulum (RER) of α1AT-producing cells (*9–12*). The α1AT-producing cells of such individuals have normal levels of α1AT mRNA (*13, 14*), but a marked reduction in the secretion of α1AT, and an accumulation of α1AT with high mannose–type carbohydrate side chains in the RER (*15, 16*). Consistent with these observations, liver biopsies of Z homozygotes show aggregation of α1AT in the RER of hepatocytes (*17, 18*). Although the mechanisms of the accumulation of the Z-type α1AT has not been completely elucidated, the crystallographic structure of the protein predicts that Glu^{342} in β-sheet A strand 5 normally forms a salt bridge link with nearby residue Lys^{290} in β-sheet A strand 6 (Fig. 1), thus helping the newly synthesized α1AT molecule to fold into its three-dimensional configuration (*19*). It has been suggested that the inability to form this salt bridge in Z mutants results in the accumulation of α1AT, in other words, the mutation at Glu^{342} impedes normal intracellular processing of the α1AT, and consequently interferes with its secretion (*19*).

We hypothesized that the Z-associated α1AT "deficiency" state might be prevented by reestablishing the salt link through modification of a second residue in the α1AT molecule. Theoretically, this second mutation would reconstitute the charge balance between residues 342 and 290 by changing the negative charge at residue 290 to a positive charge (Fig. 1). Three α1AT gene variants were constructed by means of oligonucleotide site-directed mutagenesis with a full-length human α1AT cDNA: a normal cDNA, a cDNA with the Z mutation, and a cDNA with the critical Z mutation at residue 342 and the additional mutation ($Lys^{290} \rightarrow Glu^{290}$). The cDNAs were inserted into a eukaryote expression vector which was then used to transfect and direct COS I cells (an SV40-transformed African Green monkey kidney line) to transcribe, translate, and secrete the three forms of human α1AT.

As expected, similar levels of α1AT mRNA transcripts were observed in COS I cells transfected with normal, Z, or Z-charge reconstituted (Z-CR) α1AT variants (Fig. 2A). In six experiments, no significant differences were observed in mRNA content ($P > 0.3$) as measured by a one-way analysis of variance in a multiple comparisons procedure. All three variants secreted a protein of normal size (52 kD) that was immunoprecipitated with rabbit antibody to human α1AT and migrated with human α1AT (Fig. 2B). However, the cells transfected with the Z variant cDNA secreted only 26 ± 4% ($\bar{X}$ ± SEM) of the α1AT secreted by the cells transfected with the normal variant ($P < 0.005$). Thus, the single mutation in the Z cDNA variant was sufficient to reproduce the marked reduction in secretion of α1AT comparable to that seen in α1AT-producing cells of individuals homozygous for the Z-type α1AT deficiency. In contrast, when the COS I cells were transfected with the Z-CR variant, the transfected cells secreted 93 ± 15% of the amount of α1AT secreted by cells transfected with the normal cDNA despite the presence of the Z mutation at residue 342 ($P > 0.3$, compared to normal). Thus, the addition of the second mutation at residue 290 obviated the molecular pathology associated with the Z mutation. Although an insufficient amount of the Z-CR protein was available to evaluate its function as an inhibitor of neutrophil elastase, we predict that the function of the protein should not be altered, because the second mutation was placed at a site far

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