Structural Differences in the Minimal Catalytic Domains of the GAP-activating Proteins p120GAP and Neurofibromin

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The kinetic properties for the enzymatic stimulation of the GAP reaction of p21ras by the two GAP-activating proteins (GAPs) p120GAP and neurofibromin are different. In order to understand these differences and since crystallization attempts have only been successful with truncated fragments, structure/function requirements of the catalytic core of these proteins were investigated. Differences in size of the minimal catalytic domains of these two proteins were found as determined by limited proteolysis. The minimal catalytic domain has a molecular mass of 30 kDa in the case of p120GAP and of 26 kDa in the case of neurofibromin. Both catalytic domains contain the homology boxes as well as the residues perfectly conserved among all RasGAPs. The N-terminal part of the minimal p120GAP domain is 47 residues perfect conserved among all RasGAPs and the C-terminal part of the minimal p120GAP domain is 47 amino acids longer. Thus, newly identified minimal catalytic fragments were as active in stimulating GAP activity toward p21ras as the corresponding larger fragments GAP-334 and NF-1-333 from which they had been generated via proteolytic digestion. Recently it was postulated that a fragment of 91 amino acids from neurofibromin located outside the conserved domain contains catalytic activity. In our hands this protein is unstable and has no catalytic activity. Thus, we believe that we have defined the true minimal domains of p120GAP (GAP-273, residues Met714-His986) and neurofibromin (NF-1-230, residues Asp248-Phe247), which can be expressed via LMM fusion vectors in Escherichia coli and isolated in high purity.

The product of the ras protooncogene, p21ras, is involved in a multitude of signal transduction pathways through which the binding of an extracellular signal molecule to a tyrosine kinase receptor is transmitted into the nucleus of the cell to induce transcription of specific genes. Like any other guanine nucleotide-binding protein, p21ras exists in two different conformations, an active GDP-bound form and an inactive GTP-bound form (1, 2).

In the cell the conformational state of guanine nucleotide-binding proteins is regulated by two kinds of interacting molecules, guanine nucleotide exchange factors and GAP activities (called GAPs).1 Three specific GAPs for p21ras have been described. The first, p120GAP, is the prototype of this class of proteins and was the first one to be isolated (3–5). The second is neurofibromin (NF1), which is the product of the neurofibromatosis type I gene (6) and has also been shown to stimulate the GAP activity of p21ras (7–9). This gene has been found to be frequently mutated in patients with the disease neurofibromatosis type I (10–12) but also, albeit less frequently, in solid tumors (13). A mammalian homologue (GAP1) of the Drosophila GAP1 gene (14) has been described as the third form of Ras GAP (15). Recently, a fourth member of the RasGAP family (GAP1P4BP) has been isolated, which performs GAP-stimulating activities toward both Ras and Rap (16).

p120GAP and NF1 protein can be distinguished with respect to their catalytic properties (7, 17, 18). p120GAP increases the GAP activity of p21ras more than 104-fold, kcat = 19 s−1, with a Km of 9.7 μM for p21rasGTP (18), whereas NF1 protein has been reported to have a lower kcat of 1.4 s−1 but a higher affinity (Km of 0.3 μM; 17). In studies with N-Ras the difference in affinity was similar, but much smaller in kcat (19). Certain lipids were found to inhibit the GAP activity of NF1 protein at micromolar concentrations without having an effect on p120GAP activity (20, 21), although the latter binds membranes in a Ca2+-dependent fashion in vitro (22).

Furthermore, it is likely that the biological roles of these proteins are different: p120GAP, in addition to its catalytic activity, contains several other independent functional domains such as SH2, SH3, pleckstrin homology domain, and Ca2+-dependent phospholipid binding domain (for a review, see Refs. 23 and 24), all of which seem to be involved in interactions with other signaling molecules. However, no obvious sequence correlation with other signaling molecules outside the catalytic domain has yet been identified in the primary sequence of NF1. There is some evidence that p120GAP might be more than just an inactivator of Ras and may in fact be an effector (25–27).

NF1 and GAP1m, on the other hand, seem to be more negative regulators of Ras, since, outside the catalytic domain, they display a high homology to the Saccharomyces cerevisiae IRA gene products and to the Drosophila melanogaster protein GAP1, which have genetically been found to be negative regulators of the Ras signal transduction pathway (14, 28, 29). In agreement with the concept of a negative Ras regulator, embryonic neurons from NF1 knockout mice survive in the absence of neurotrophin, which signals via Ras (30). In addition, the NF1 gene has characteristics of a tumor suppressor gene, as evidenced from the abnormal regulation of p21ras activity in neurofibromatosis type I patients (31, 32).
To obtain information on the mechanism of stimulation of GAP activity we have set out to crystallize the minimal catalytic domain of Ras GAPs. Originally, a 483-residue GAP-related domain of neurofibromin had been described to contain full Ras GAP activity (7). Later, smaller fragments of between 333 and 343 residues were shown to possess full or almost full Ras GAP activity (18, 33, 34). Recently, Maruta and co-workers (35) reported that 91- and 78-residue fragments from the NF1 protein located outside the conserved RasA domain (see Fig. 1) can still stimulate the GTPase activity of Ras and that even smaller fragments of 56 amino acids from the same region still binds to Ras-GTP (36). These reports together with the results of crystallization trials prompted us to reinvestigate the properties of the catalytic domains of GAPs and to rigorously define their boundaries.

MATERIALS AND METHODS

Fragment Cloning—For the bacterial expression of NF1 fragments, the plasmid ptrNF1–1–333, which directs the synthesis of the 333-amino acid sequence between positions Glu1368 and His1570, was used as starting material (17). We have also created a new expression plasmid, pETNF1–333, which expresses the same fragment from a T7 promoter, by redacting a NcoI/HindIII fragment from ptrNF1–1–333 into a modified pET-3d vector (37).

Using the polymerase chain reaction (PCR) we constructed various expression plasmids for the NF1 catalytic domain, which are further shortened at the N terminus (18N, 32N and 66N) and/or the C terminus (54C and 96C) as indicated by the corresponding number of deleted amino acids. The pET vector was used for the 18N, 32N, 46N, and 66N deletions. The oligonucleotides used for the constructions were as follows: 18N, 5'-AGGAGGCCATGGGTGATCAAGGAGAACTCCCT-3'; 32N, 5'-AGGAGGCCATGGTTCTCTGTTTGATTCTCGGCAT-3'; 46N, 5'-AGGAGGCCATGGTACTCTGTTGATTCTCGGCAT-3'; 66N, 5'-AGGAGGCCATGGTACTCTGTTGATTCTCGGCAT-3'.

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Expression plasmids for the NF1 catalytic domain, which are further shortened at the N terminus (18N, 32N, 46N, and 66N deletions), and/or the C terminus (54C and 67C) were expressed by recloning a pET vector into the corresponding vectors (ptrc99A- and pET-3d-modified). For the NF1–230 fragment, NF1–230 was produced by IgA-protease digestion, different concentrations of p120GAP and 26-kDa fragment resulting from chymotrypsin digestion of NF1–333 was separated from the protease by chromatofocusing on polybuffer exchange PBE 94 (Pharmacia), applying a pH gradient from pH 5.8 to 8.0, 200 mM KCl, 1 mM EGTA, 5 mM DTE, and 0.04% NaN3. The polypeptide was dialyzed against buffer B, and the polybuffer was removed by gel filtration on Superdex as described for the clostripain fragment (see below).

The reaction mixture from preparative digestion of GAP-334 by polypeptide K was applied to a Pharmacia Mono-Q HR 5/5 column and eluted with a 20-ml linear gradient of 0-200 mM NaCl in buffer A at a flow rate of 1 ml/min. The major peak at 130 mM NaCl was pooled. Protein concentrations were determined following the method of Bradford (41) using bovine serum albumin as standard.

Protease Digestions—Purified NF1–333 or the fusion protein LMMNF1–40 (40) were treated with the indicated proteases at a substrate protease ratio of 100:1 (w/v) at room temperature. The buffer for digestion with clostripain contained 20 mM MgCl2, 10 mM DTE buffer or 20 mM triethanolamine/HCl (pH 8.0). For the digestion of the LMMNF1 fusion protein 600 mM KCl was included in the buffers, since it has been shown that LMM fusion proteins retain the property of intact LMM of being soluble only at high KCl concentrations. The digestion time was up to 2.5 h for NF1–333 and GAP-334 and up to 12 h for the LMMNF1 fusion protein. The reaction was terminated with 50 μg/ml leupeptin for the trypsin reaction, 1 mM phenylmethylsulfonyl fluoride for chymotrypsin and protease K, and 20 mM EGTA for clostripain. The digestion of 3.4 μM NF1–333 with protease K was done in the presence or absence of 11.5 μM p21(Q61L)Gpp(NH)p. Aliquots from the proteolysis reaction were withdrawn at the indicated time points, the reactions were terminated, and aliquots were analyzed either for GAP activity as described above or for protein content on SDS-polyacrylamide gels.

27 μM GAP-334 in the presence or absence of 182 μM truncated residues (1–66) p21(Q61L)Gpp(NH)p was digested at room temperature with protease K or subtilisin Carlsberg at a substrate protease ratio of 100:1 or 20:1 (w/v) in 40 mM Hepes/NaOH (pH 7.5), 2 mM MgCl2, 10 mM DTE buffer or 20 mM triethanolamine/HEPES (pH 8.0), 5 mM DTE for preparative purposes. p21(Q61L)Gpp(NH)p is calculated to complex 95% of GAP-334, taking a dissociation constant of 5 μM.2 The reaction was terminated with 2.5 mM phenylmethylsulfonyl fluoride and analyzed by GAP activity measurements or SDS-polyacrylamide gel electrophoresis.

Protein Sequencing—Proteins and the proteolytic fragments thereof were sequenced on an Applied Biosystems A470 gas-phase sequencer, and the resulting phenylthiohydantoins were identified and quantified off line.

GAP Activity Measurements—GTPase assays were done as described previously (4, 43) by loading p21(Q61L)GTP (44) with [γ-32P]GTP. Excess nucleotide was removed on a NAP-5 gel filtration column (Pharmacia) in the GAP reaction buffer (20 mM Hapes/NaOH (pH 7.5), 1 mM DTE). In the presence of the appropriate amount of GAP/NF1 protein and 2 mM MgCl2 the GTPase reaction was measured by following the decrease of [γ-32P]GTP bound to p21(Q61L) as determined by a nitrocellulose filter-binding assay. Under standard conditions for the determination of the specific activities of different NF1 preparations, the starting p21(Q61L)GTP concentration was 1 μM. The decrease of radioactivity over time was fitted by a single-exponential decay function using the program Enzfitter (Elsevier Biosoft). For the determination of the enzymatic properties (kcat and km) of NF1 fragments, a constant amount of NF1 was treated with increasing concentrations of p21(Q61L)GTP using different reaction volumes such that similar numbers of radioactivity counts were retained on the filters. In the case of the 26-kDa NF1 fragment produced by chymotrypsin digestion 4 μM enzyme was added to 121, 242, 362, 604, 1210, and 1810 μM p21(Q61L)GTP in GAP reaction buffer, respectively. In the case of NF1–1–230 produced by IgA-protease digestion, different concentrations

2 M. R. Ahmedian, unpublished observations.

3 A. Lautwein, unpublished observations.
of enzyme (0.5, 1, 2, and 5 nM) were reacted with 0.1, 0.2, 0.3, 0.5, 0.8, 1, 2, and 3 μM p21ras[−32P]GTP in GAP reaction buffer. The initial linear portion of the reaction was used as the initial rate of the reaction. The error of these measurements was estimated to be 15%. For the determination of $K_m$ and $k_{cat}$ values, the initial rates measured with increasing concentrations of p21ras substrate were fitted directly to the Michaelis-Menten equation using the program Enzfitter.

RESULTS

Deletion Analysis of the NF1 Catalytic Domain—We have shown earlier that a fragment of the NF1 protein containing 333 amino acids from position Glu1198 to His1530 (17) (see Fig. 1) can be expressed in E. coli using the trc promoter and that this recombinant protein, NF1–333, can be purified using a three-column procedure. From preparations of this catalytic fragment we obtained crystals that appeared to be suitable for x-ray crystallography. Gel electrophoretic analysis of these crystals showed, however, that they contained an NF1 fragment of a much lower molecular mass compared with the one representing the predominant species in the preparation used for the crystallization set-up (Fig. 2). The small fraction of this fragment that could be solubilized from crystals appeared to be catalytically active (not shown). This was surprising with respect to earlier results with p120GAP, which showed that a fragment comprising 343 amino acids could not be truncated further without loss of catalytic activity or solubility after expression in E. coli (33). In contrast to this report, Nur-E-Kamal et al. (35) postulated Ras GAP activity for a 78-amino acid fragment of NF1 located outside the motifs conserved among RAS-GAPs. This prompted us to investigate more closely...
whether smaller fragments of NF1 and GAP could be generated that would retain full GTPase activating function toward p21<sup>ras</sup>. 

Fig. 1 shows an alignment of various Ras GAP sequences. Boxes of homologous sequences reported earlier (9, 45, 46) are indicated. We have made various deletion constructs by shortening NF1–333 at the N and/or C terminus as indicated (Fig. 1). Based on the molecular mass, in kDa, as indicated.

![Figure 2](image)

**Fig. 2. Results from crystallization experiments showing that the protein NF1–333 used for crystallization trials was accidentally digested to smaller fragments during the purification procedure, probably by contaminating proteases.** An SDS-polyacrylamide gel containing various protein samples was silver-stained. Lanes 1–4 contain NF1–333 (3), mother liquor of crystallization setup (2), dissolved crystal (3), and the proteinase K fragment (4), the fragment from the experiment presented in Fig. 3, respectively. Lane M contains markers with molecular masses, in kDa, as indicated.

For the localization of the C-terminal end the molecular mass of the chymotryptic fragment was measured by laser desorption mass spectroscopy from two independent preparations. In the first case a single mass peak of 25,961 Da was obtained, which is very close to the calculated molecular mass (25,951 Da) of a 226-amino acid fragment starting at Asp<sup>1248</sup> and ending at His<sup>1473</sup>. In the second case three closely related mass peaks were found with a mean molecular mass of 26396 Da. This would indicate that Phe<sup>1477</sup> is the C-terminal amino acid rather than His<sup>1473</sup>. Cleavage after Phe<sup>1477</sup> is more likely, considering the specificity of chymotrypsin cleavage. The specific activity of the chymotryptic fragment amounted to 67% of the activity of NF1–333.

The stable fragment after chymotrypsin digestion, whose apparent molecular mass of 26 kDa was close to that seen in the crystal, was further characterized after preparative polyacrylamide gel electrophoresis of LMMNF1. Purification via chromatofocusing and gel filtration removed the LMM portion, the smaller peptides as well as the protease, as described under “Materials and Methods.” The purified fragment was subjected to protein sequencing. From the first 19 amino acids 17 were unequivocally identified as DSRHLYQQLXNMFXKEVE, implying that chymotrypsin cleaved behind Phe<sup>1477</sup> and removes 50 amino acids from the N terminus of the NF1–333 protein as indicated (Fig. 1).

![Table I](image)

**Table I** GTase-activating properties of various proteolytic fragments of NF1. The apparent molecular masses were determined by SDS-gel electrophoresis. The exact molecular mass of the chymotryptic fragment is 26396 Da, determined by mass spectroscopy as described in the text. The 100% activity value resembles 1575 units/mg (17).

| NF1 fragments after digestion | mol. mass | Specific activity |
|-----------------------------|----------|------------------|
| NF1–333                     | 34       | 100              |
| Clostripain                 | 32       | 101              |
| Trypsin<sup>a</sup>          | 30       | 70               |
| Chymotrypsin                | 26       | 67               |
| Proteinase K<sup>a</sup>     | 26       | 81               |

<sup>a</sup>In these cases the specific activity of the proteolytic fragments was determined in the digested sample before further purification using the conditions as described under “Materials and Methods.”

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81% of the wild-type activity (Table I). Unfortunately, it turned out to be unsuitable for purification (and crystallization) because of continued proteolysis and a strong tendency to aggregate as seen on a native polyacrylamide gel.

Having established the minimal 230-residue fragment from NF1 by chymotrypsin cleavage we utilized improved LMM expression vectors with various specific protease sites to test whether we could express this domain as a LMM fusion protein. Fig. 4 illustrates that the 230-residue fragment of NF1 (Asp1248 to Phe1477) could indeed be isolated using an LMM expression vector with an IgA protease cleavage site. The protein could be isolated as a fusion protein, released from LMM by IgAse proteolysis and isolated in large amounts (currently used to grow crystals of the catalytic domain of NF1). The enzymatic properties of NF1–230 were determined by Michaelis-Menten analysis (Fig. 5) to obtain a $K_m$ of 0.65 M and a $k_{cat}$ of 7.3 s$^{-1}$. In comparison with the activity of the chymotryptic product from NF1–333 the catalytic activity of the expressed NF1–230 is 8-fold higher. This can be explained by the improved preparation method of NF1–230 using the LMM vector. NF1–333 prepared from the corresponding vector has similar catalytic properties.

Minimal Fragment of p120GAP — A 227-residue fragment from p120GAP (Leu761–Asp989), which according to sequence alignment corresponds to NF1–230 (Fig. 1), could be expressed as an LMM fusion protein in large amounts but was insoluble (data not shown). This prompted us to investigate the catalytic activity of GAP–334 after proteolysis. Fig. 6 shows the progressive digestion of GAP–334 with proteinase K. GAP–334 proteolysis produces a final fragment of an estimated 30-kDa molecular mass, which is stable against further degradation.

In parallel, GAP–334 was identically treated in the presence of saturating amounts of p21(Q61L)Gpp(NH)p, which binds to GAP–334 with a dissociation constant of 5 $\mu$M, as compared with 19 $\mu$M for wild type p21$^{ras}$. Thus, like full-length p120GAP, GAP–334 has a higher affinity for the Gpp(NH)p complex of p21(Q61L), although the difference between wild type and mutant is more pronounced with full-length p120GAP (0.1 $\mu$M versus 4 $\mu$M dissociation constants, respectively; Refs. 20 and 18).

As with NF1–333, the addition of p21(Q61L) considerably reduces the half-life of GAP–334 to about 2 h (Fig. 6B). The final fragments produced have identical molecular masses (30 kDa) in the presence and absence of complex formation with p21(Q61L)Gpp(NH)p. Therefore, we conclude that in the GAP–334 complex, regions of GAP–334 are less accessible for proteolytic digestion and can only be cleaved after dissociation of the complex (Fig. 6).

The proteinase K fragment of GAP–334 was purified by ion exchange chromatography. By protein sequencing of the proteinase K fragment of GAP–334, 8 of the 14 N-terminal amino acids could be unequivocally identified and turned out to be identical to those of undigested GAP–334 (Fig. 1). Upon molecular mass determinations of the proteinase K by laser desorption mass spectroscopy, one major and two minor mass peaks were consistently found. This can be explained by heterogeneities in the original GAP–334 protein preparation, although the polypeptide subpopulations must have similar molecular weights, as they could not be visibly resolved on an SDS-polyacrylamide gel. The masses of the major and minor fragments were 31216, 32553, and 30178 Da, respectively. The dominant fragment mass is close to the calculated mass for the 273-amino acid-long GAP peptide with His$^{989}$ at the C terminus (Fig. 1). The minor peak at 32,553 Da probably corresponds...
to a protein that became visible in the SDS gel only after protease digestion (apparent molecular mass, 35,000 Da). It may be unrelated to GAP-334, but it co-chromatographed.

The 273-residue fragment from p120GAP from Met714 to His986, corresponding to the proteinase K cleavage product, could be expressed in E. coli as a LMM fusion protein. Fig. 4B shows that the protein was isolated as LMM fusion protein, cleaved from LMM by IgA-protease, and separated from LMM by dialysis against low salt buffer. It remained soluble after purification. Since it had been reported that an NF1 fragment NF1–91—a 26-kDa fragment described here, NF1–333 (17, 19), NF1-GRD with 483 amino acids (7), and the complete NF1 protein containing 2818 amino acids (48) have apparently similar enzymatic properties. By improving the purification procedure for NF1 fragments using LMM fusion vectors (40) it now appears that the maximum rates of the GTPase reactions (kcat) catalyzed by neurofibromin and p120GAP may in fact be very similar, in agreement with data from Eccleston et al. (19). These authors used increasing concentrations of GAP-344 to determine kcat rather than increasing concentrations of substrate, whereby the measurement becomes independent of the quality of the GAP-344 preparation.

What is the basis of the different properties of p120GAP and neurofibromin? Is there a recognizable structural difference between the catalytic domain of the two proteins? A partial answer to this question is given by the results presented in this paper concerning the dimensions and stability of the catalytic GTPase-activating domains of these proteins. Ballester et al. (9) and Wang et al. (45) applied the program MACAW (47) to locate, analyze, and assess the statistical significance of regions of local similarity between the sequences of the Ras GTPase-activating proteins (see also Ref. 46). We have extended these studies to include some recently cloned GAP genes (some of which are shown in Fig. 1). The sequences contain four blocks of similarity within the catalytic domains of these proteins. NF1, IRA1, and IRA2 display additional sequence similarities outside the catalytic domain (not shown). The sequence alignment depicts the 12 residues that are totally conserved and many other residues that are almost completely conserved. The proteolysis experiments together with the corresponding enzymatic activity measurements presented in this study show that the sequences in the homology boxes, and all the totally conserved amino acids are indeed necessary for structural and/or enzymatic integrity of at least the two Ras GAPs investigated here. Only two of the highly conserved residues in the N terminus of the catalytic domain (Ala1226 and Leu1243 of NF1) are missing in the minimal catalytic domain of NF1.

Our data show that the minimal, catalytically active domain of p120GAP, which is reasonably stable against further proteolysis, is 47 amino acids longer than NF1–230 at the N terminus, while possessing the same C terminus. Since these additional 47 amino acids of GAP-334 cannot be cleaved from the
catalytic domain, this part of the protein adopts a different conformation in p120\(^{\text{GAP}}\) and NF1. Another indication of the structural importance of the N-terminal amino acids of the catalytic domain of GAP-334 comes from the observation that GAP-227, which corresponds to NF1-230, cannot be folded correctly in bacteria and is therefore insoluble. It is very likely that the overall three-dimensional structures of the catalytic domains of p120\(^{\text{GAP}}\) and NF1 will be similar. The difference in structure may thus be confined to the N-terminal part of the polypeptide chain. This might either adopt a different secondary structure or, in the case of p120\(^{\text{GAP}}\), might be characterized by stronger interactions with the rest of the domain, thereby preventing access to the protease.

It is interesting to note that the differences in enzymatic characteristics and structure are related to different biological roles for p120\(^{\text{GAP}}\) and NF1. Mostly due to the extensive sequence similarity with the S. cerevisiae IRA gene product and to the absence of SH2 and SH3 domains, the NF1 protein might be primarily considered a negative regulator that keeps the absence of SH2 and SH3 domains, the NF1 protein might be considered a negative regulator that keeps the conformation in p120\(^{\text{GAP}}\) and NF1. Another indication of the characteristics and structure are related to different biological roles. Max-Planck-Institut für medizinische Forschung in Heidelberg.

Ken Holmes for support during the part of the work conducted at the Max-Planck-Institut für medizinische Forschung in Heidelberg.

Patricia Stege and Anna Scherer for expert technical assistance, and Wood (Hoffmann-La Roche) for the mass spectroscopic analysis.

A complex between NF1–230 and p21

We do not have any explanation for this discrepancy.

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