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Characterization of a Gly19→Val mutant of *ram* p25, a low *Mr* GTP-binding protein: Loss of GTP/GDP-binding activity in the mutated *ram* p25

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Summary: A substitution of Gly for Val at position 19, which corresponds to oncogenic Gly13→ Val mutation of *ras* p21, was introduced in a low *Mr* GTP-binding protein, *ram* p25. The protein was expressed in cytosolic fraction of *Escherichia coli* and purified by using specific antibody raised against *ram* p25. The mutated protein had no guanine nucleotide-binding activity although [Val13]*ras* p21 was reported to have. The analysis of guanine nucleotide composition of the purified [Val19]*ram* p25 revealed that the protein was free of nucleotide whereas the normal *ram* p25 bound about 1 mol of GDP per mol of protein. These results strongly suggested that some part(s) of variable regions as well as the consensus regions are important for the biochemical properties of *ram* p25.

There is a superfamily of *ras* p21-like low *Mr* GTP-binding proteins. The *ras* proteins (Ha-, Ki-, N-*ras* p21s) have been implicated in pathogenesis of human cancer, based on the observation that a large proportion of tumors contains mutated *ras* genes possessing the potential to oncogenically transform tissue culture cells (1, 2). In eukaryotic cells, a variety of *ras*-related low *Mr* GTP-binding proteins have been described that might regulate various cellular activities (3-5). In mammalian cells, such low *Mr* GTP-binding proteins exhibit 20 - 50% homologies with *ras* p21. These proteins share structural and biochemical properties that resemble those of heterotrimeric G proteins involved in signal transduction across the cellular membranes (6, 7).

The *ram* gene encodes a GTP-binding protein with a *Mr* of 25,068 (*ram* p25) which shares about 23% homology with Ha-*ras* p21 (8). It is notable that its putative effector domain is very similar to that of yeast *SEC4* protein (9), and *ram* p25 shares 40% identity

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Abbreviations used are: G protein, GTP-binding protein as a transducer in transmembrane signal transduction; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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and 60% homology with *SEC4* protein. When *ram* cDNA was expressed in *E. coli* and its product was then purified by using several column chromatographies, it had GTP/GDP-binding and GTP-hydrolysis activities (10).

In the present study, we have introduced a point mutation, Gly19→Val, corresponding to Gly13→Val substitution of Ha-*ras* p21 in *ram* p25, and purified the mutant *ram* p25 ([Val19]*ram* p25) from the soluble fraction of an overproducing *E. coli* by using polyclonal antibody and characterized it. The unique properties of [Val19]*ram* p25 was compared with those of the corresponding oncogenic [Val13]*ras* p21, which was recently investigated (11).

MATERIALS AND METHODS

Materials and chemicals

Expression vector with T7 promoter, pHL2, and the host strain of *E. coli*, BL21 (DE3) (12) were gifts from Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan). Synthetic oligonucleotide was kindly supplied by Dr. T. Takeda (University of Tokyo, Tokyo, Japan). [^{35}S]GTPγS (specific activity, 1400 Ci/mmol), [α-^{32}P]dCTP (specific activity, 3000Ci/mmol) were from Du Pont-New England Nuclear. Site-directed mutagenesis kit "Mutan K" was from Takara Shuzo Co., Ltd. Other materials and chemicals were obtained from commercial sources. The antibody, tentatively termed Ab-*ram*1, was raised against a synthetic peptide (^{77}GQERFRSLTTAFFRD^{91}) of *ram* p25+.

In vitro mutagenesis

Site-directed mutagenesis was performed using "Mutan K" kit according to the manufacturer's instruction. Sequence of the mutant gene was confirmed by the dideoxy chain termination method (13) in the presence of [α-^{32}P]dCTP. The construction of [Val19]*ram* p25 was performed by the same method as described (10) using a 19 base-oligonucleotide 5'GAGACTCTGTAGTGGGGAA3' (mismatching nucleotide is indicated by underline). Expressed *ram* p25s were purified essentially as described (10).

Determination of the guanine nucleotide content of *ram* p25s

The nucleotide content of [Val19]*ram* p25 was determined by the method as described (14).

[^{35}S]GTPγS-binding and GTPase assay

[^{35}S]GTPγS-binding activity was determined by the rapid filtration technique as described (15).

Tryptic digestion of *ram* p25s

Normal and [Val19]*ram* p25 purified partially from *E. coli* (150μg/ml) were digested at 25℃ with 3μg/ml of tosylphenylalanyl chloromethyl ketone-treated β trypsin in 100μl of reaction mixture containing 20mM Tris/HCl (pH 7.5) and 1 mM EDTA. At the indicated times, aliquots (15μl) of the reaction mixture were removed and mixed with 3μl of soybean trypsin inhibitor (50μg/ml) containing 10% SDS and 50mM DTT. The samples were subjected to SDS-PAGE followed by immunoblotting.

Other assays

SDS-PAGE was performed accordingly to Laemmli's methods (16), and proteins were visualized with Coomassie blue stain. Immunoblot analysis was performed essentially as described (17). Protein was determined with Bio-Rad protein assay (18) using bovine serum albumin as a standard.

RESULTS

Expression and purification of the mutated *ram* and *ras* genes in *E. coli*

[Val19]*ram* p25 and normal *ram* p25 were expressed separately in *E. coli* in a time-dependent manner (10) and the cells were then disrupted by sonication in the presence of

+Characterization of the Ab-*ram*1 will be described elsewhere.

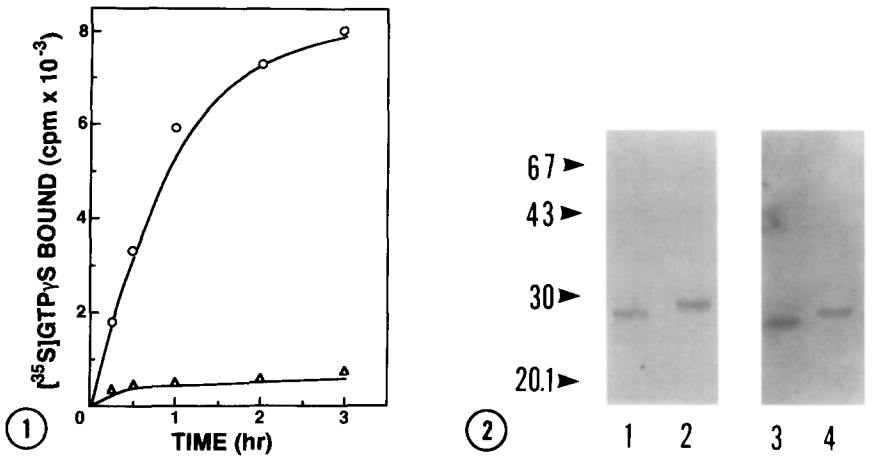

Fig. 1. Time-course studies of [^{35}S]GTPγS-binding activity of the normal and [Val19] *ram* p25s

At time points (zero, 30min, 1hr and 2hr) after induction of normal and [Val19]*ram* p25s, soluble protein fractions were prepared. The aliquots of the soluble protein (5 μg) at the indicated time points were assayed for [^{35}S]GTPγS-binding activity. ○, normal *ram* p25; △, [Val19]*ram* p25. The results shown are the representative of two independent experiments.

Fig. 2. SDS-PAGE and immunoblot analyses of recombinant normal and [Val19]*ram* p25s

Purified normal and [Val19]*ram* p25 samples (0.5μg) were subjected to SDS-PAGE on 15% gel followed by immunoblot analysis with Ab-*ram*1. *Lane 1 and 3*, normal *ram* p25; *lane 2 and 4*, [Val19]*ram* p25. *Lanes 1 and 2*, SDS-PAGE analysis. *Lanes 3 and 4*, immunoblot analysis. The protein markers (Pharmacia) used were bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000) and trypsin inhibitor (20,100).

5mM $MgCl_2$ followed by centrifugation at 105,000 x g for 90min. When the [^{35}S]GTPγS-binding activity for the cytosolic fraction of containing normal and the mutated *ram* p25s was measured, the former fraction showed a drastic increment of the binding activity in a time-dependent manner but the activity for the latter was undetectable (Fig. 1). The corresponding mutant of *ras* p21 ([Val13]*ras* p21) had the binding activity as described (11). Thus, it was thought that *ram* p25 lost the guanine nucleotide-exchange activity by the mutation. Moreover, it had no activity to bind [α-^{32}P]GTP and [^{3}H]GDP under the conditions in which normal *ram* p25 showed the binding activities (data not shown). Thus, we purified the protein by an antibody Ab-*ram*1 using successive column chromatographies of DEAE-Toyopearl 650(S), hydroxyapatite HCA-100S and MonoQ HR5/5 (data not shown). $MgCl_2$ concentration was adjusted to 5 mM at every purification steps, under which condition GDP bound to *ram* p25 dissociates very slowly. The final preparation of [Val19]*ram* p25 was shown in Fig. 2.

Limited tryptic digestion of *ram* p25

ram p25 and [Val19]*ram* p25 were digested with trypsin and then proteolytic fragments were analyzed by SDS-PAGE followed by immunoblot analysis using the Ab-*ram*1 (Fig. 3). The limited fragmentation patterns of [Val19]*ram* p25 were quite different from those of normal *ram* p25. The mutant was trypsin-resistant and was not digested even after 90min

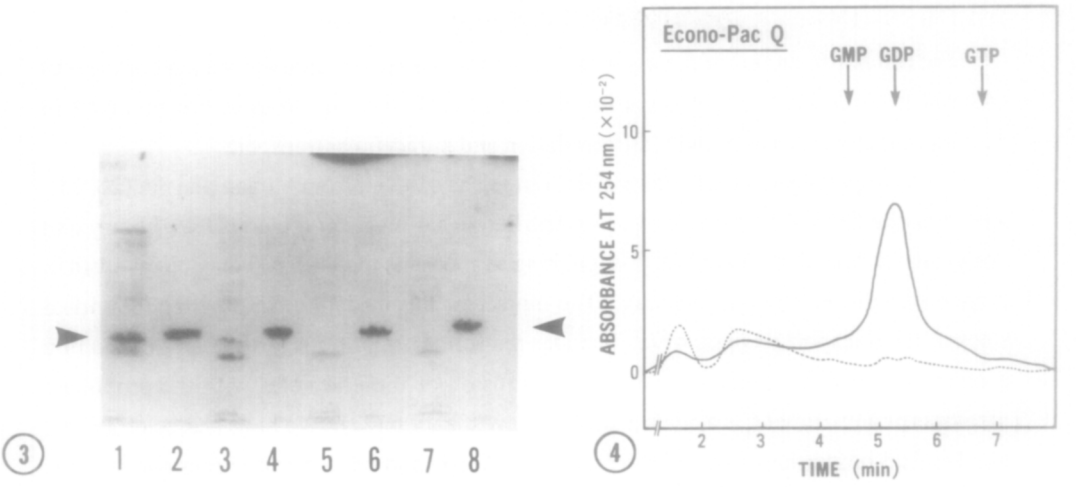

Fig. 3. Limited tryptic digestion of normal and [Val19]*ram* p25s
Normal and [Val19]*ram* p25s were subjected to limited proteolytic digestion with trypsin as described under "Materials and Methods". At the indicated times, aliquots of the digestion mixture were removed and applied to each lane of SDS-polyacrylamide gel (15%). *Lanes 1, 3, 5 and 7*, normal *ram* p25; *lanes 2, 4, 6 and 8*, [Val19]*ram* p25; *lanes 1 and 2*, zero min; *lanes 3 and 4*, 0.5min; *lanes 5 and 6*, 2min; *lanes 7 and 8*, 5min. *Left and right arrows* indicate normal and the mutant *ram* p25s, respectively.

Fig. 4. Identification of guanine nucleotide bound to normal and [Val19]*ram* p25s
The identity of the guanine nucleotide found in the normal *ram* p25 filtrate was determine chromatographically as described in "Materials and Methods". The authentic samples of GMP, GDP and GTP were separated by Econo-Pac Q column as indicated with *arrows*. The elution profiles of the authentic samples were not altered by boiling before application. —, normal *ram* p25; ···, [Val19]*ram* p25.

incubation, although normal *ram* p25 was digested considerably at 30sec and completely at 10min. Other mutants of *ram* p25 (Thr41→Ser, Ala76→Thr, Gln78→Leu, Asn133→His) were also digested in the same manner as that of normal *ram* p25 (data not shown).

Determination of the guanine nucleotide content of purified *ram* p25s

Like *ras* p21, *ram* p25 and some of their mutants produced in *E. coli* were isolated as stable GDP-bound proteins (10, 19). To ascertain whether the purified [Val19]*ram* p25 also contained guanine nucleotide, the nucleotide content of the protein was investigated. After denaturation of purified normal and [Val19]*ram* p25s by boiling and removing the proteins by centrifugation using Centricon 10 (Amicon), absorbance at 254 nm was measured for each of filtrates. Normal *ram* p25 was found to contain about 1 mol of nucleotide per mol of protein and the chromatographic analysis of the protein by using Econo-PacQ column (Bio-Rad) revealed that the released nucleotide was GDP (10). Thus, the normal *ram* p25 bound about 1 mol of GDP per mol of protein. On the other hand, the chromatographic pattern of the filtrate of [Val19]*ram* p25 was almost the same as that of the buffer, and no peak corresponding to either GDP or GTP was observed for [Val19]*ram* p25 (Fig. 4), indicating that the mutant was nucleotide-free form whereas the corresponding mutant of *ras* p21 bound GDP (11). [Val13]*ras* p21 showed considerably increased dissociation rates 80-fold and 6-fold for GDP and GTP, respectively (11).

DISCUSSION

The cellular functions of the mammalian *ras* and the related proteins remain elusive, but increasing evidence strongly suggests that these GTP-binding proteins are involved in cellular processes such as growth, differentiation and intracellular transport.

We have purified a *SEC4*-like protein, termed as c25KG, from human platelet (20, 21), and then one *SEC4*-like gene has been identified in rat megakaryocyte cDNA library and designated as *ram* (8). Therefore, it is tempting to speculate that *ram* p25 may be involved in certain step of the exocytic pathway. The molecular mechanism governing the individual steps of the transport process, which includes the formation of transport vesicles containing proteins destined for export and the directed fusion of these vesicles with the proximate membrane compartment, is a central target of investigation (22). We have characterized the biochemical properties of *ram* p25 produced by *E. coli* and baculovirus expression systems (10, 23). We also studied the effect of the Gly to Val substitution at position 19 of *ram* p25, in a consensus region, Gly-X-X-(Ala/Gly/Ser)-X-Gly-Lys-(Ser/Thr) (X is any amino acid), which is thought to be involved in GTP-hydrolysis reaction in *ras* p21. In the case of *ras* p21, by the corresponding point mutation of Gly13 to Asp, Val or Arg, it can be activated and becomes oncogenic and causes neoplastic transformation of mammalian cells (1). The mutations of Gly13 to Val or Asp were first found in human acute myeloid leukemia (24), and many other *ras* oncogenes with various Gly13 mutations have since been reported in various tumors (25). This oncogenecity of the mutated *ras* p21 is due to constitutive acceleration of guanine nucleotide exchange (11).

In the present study, we produced [Val19]*ram* p25 and compared its properties with those of normal *ram* p25 and corresponding mutant [Val13]*ras* p21. The point mutation (Gly19 →Val) of *ram* p25 apparently prevented guanine nucleotide exchange. The results raised two possiblities. One was that this mutant did not fold properly and was biochemically "dead". The other was that this mutation blocked guanine nucleotide exchange although the mutant contained prebound GDP. The latter possibility was neglected by the finding that [Val19]*ram* p25 did not bind guanine nucleotide properly when it was purified in the presence of 5mM $MgCl_2$. The reason for the discrepancy between [Val19]*ram* p25 and [Val13]*ras* p21 remains to be clarified, but the differences in the neighbouring amino acid sequences or in other partial structures may partly account for the discrepancy. Although the three-dimensional structure has not yet been investigated, it is possible that this consensus region of *ram* p25 containing Gly19 participates in guanine nucleotide binding as well as GTP-hydrolysis in *ram* p25. This supposition can be supported by the observation obtained in *YPT1* protein. Namely, when a mutation in the consensus region mentioned above, Lys21→ Met, was introduced in the consensus region of *YPT1* protein, the binding of GTP to the protein was abolished and the protein remained in GDP-bound form (26).

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