A novel human RasGAP-like gene that maps within the prostate cancer susceptibility locus at chromosome 1q25

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Abstract We report the molecular cloning of a human cDNA that encodes a molecule having striking homology with Ras-specific GTPase-activating proteins (RasGAPs). Among previously described RasGAPs, the cDNA product is most closely related to *Caenorhabditis elegans* GAP-2, including a predicted coiled-coil structure near the carboxyl terminus. Expression of the cDNA in *Saccharomyces cerevisiae* defective in one of two RasGAPs, Ira2, complemented loss of the Ira2 function, indicating that the cDNA product functions as a RasGAP. The RasGAP-like gene is located on the human chromosome 1q25, the locus that appears to contain a hereditary prostate cancer susceptible gene, *HPC1*.

Key words: Ras-specific GTPase-activating protein; nGAP; NF-1; Tumor suppressor gene; HPC1

1. Introduction

Since the first identification of Ras as a protooncogene product whose mutations are involved in the transformation of a variety of mammalian cells, accumulating evidence suggests that Ras plays crucial roles in the control of cell growth and differentiation [1,2]. Ras is a membrane-associated protein that binds and hydrolyzes GTP to GDP [3]. By acting as a molecular switch which cycles between the active GTP- and the inactive GDP-bound states, Ras is thought to function as a transducer of mitogenic signals.

The Ras-dependent signalling is controlled by two distinct mechanisms, each of which involves regulatory molecules affecting the GTP-GDP equilibrium: Ras guanine nucleotide-releasing proteins (RasGNRPs) [4] and Ras GTPase-activating proteins (RasGAPs) [3]. RasGNRPs act as positive regulators of Ras by exchanging GDP with GTP, whereas RasGAPs negatively regulate Ras activity by stimulating the weak intrinsic GTP-hydrolyzing activity of Ras.

There are four distinct RasGAPs so far identified from mammalian cells; p120GAP, neurofibromin, Gap1^{m}, and Gap1^{IP4BP} [5–10]. One of these, neurofibromin, was originally identified as a gene (*NF1*) product whose functional inactivation causes von Recklinghausen neurofibromatosis type 1 disease (NF1) [7,8]. Since NF1 is a cancer-prone disorder, neurofibromin is suspected to negatively regulate Ras-mediated mitogenic signal transduction and hence prevent cellular transformation [11,12].

RasGAPs have also been identified in lower eukaryotes. Yeast *S. cerevisiae* possesses two RasGAPs termed Ira1 and Ira2 [13–15]. Disruption of either the *IRA1* or the *IRA2* genes leads to a phenotype that resembles that seen in cells containing constitutively active Ras. In particular, *ira1*$^{-}$ or *ira2*$^{-}$ strains are highly sensitive to heat shock and cannot survive after heat shock treatment [13,14]. However, when RasGAP molecules from mammalian cells are introduced and ectopically expressed in these *ira*$^{-}$ cells, heat shock resistance is acquired [16–18]. This demonstrates that RasGAPs are structurally as well as functionally conserved throughout eukaryotic evolution. RasGAPs have been also identified in *Drosophila melanogaster* [19] and in *Caenorhabditis elegans* (*C. elegans*) [20,21]. All RasGAPs so far characterized share a highly conserved domain called GAP-related domain (GRD), which is required for the catalytic activity of RasGAP [17].

In this work, we describe the molecular characterization and chromosomal mapping of a human gene encoding a novel protein, termed nGAP, having the GAP-related domain (GRD) that is strongly conserved among RasGAPs.

2. Materials and methods

2.1. PCR-based cDNA cloning and plaque hybridization

5′-rapid amplification of cDNA ends (5′-RACE) was performed with the use of a sequence-specific reverse primer and a primer for the λZAPII phage vector sequence by polymerase chain reaction (PCR) using Taq polymerase. The PCR-amplified fragments were ligated into pCRII vector and was used as a probe to screen human heart cDNA library.

2.2. Reverse transcription-PCR analysis

Poly(A)$^{+}$ RNA (0.5 μg) from various tissues of adult ICR strain mice was reverse transcribed by AMV reverse transcriptase with cDNA Cycle Kit (Invitrogen). Subsequently, PCR was performed for 30 cycles for 1 min at 94°C, 2 min at 55°C and 1 min at 72°C, employing the oligonucleotide primers for mouse nGAP, 5′-GTACCCAGTGAGTACACCC-3′ and 5′-CTCCACAACGATCTACCTCT-3′. The PCR product was subjected to electrophoresis in a 2% agarose gel.

2.3. Plasmid construction

pKT10 is a *S. cerevisiae* expression vector which contains a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. Other plasmids were constructed from pKT10 by introducing various cDNAs; pKT18 contains a cDNA encoding Ira2-GRD (residues 1451–2255)

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[13–16]. pKP11 and pKP12 express neurofibromin-GRDs encompassing residues 1063–1672 and residues 1063–1559, respectively. pNF201 contains a cDNA encoding neurofibromin-GRD encompassing residues (1063–1672) with Phe^{1434} to Leu mutation [22]. A series of cDNA fragments encoding parts of nGAP were generated from the full length nGAP cDNA and respectively subcloned into pKT10; nGAP(298–1139) corresponding to residues 298–1139, nGAP(1–646) corresponding to residues 1–646, nGAP(251–646) corresponding to residues 251–646, nGAP(217–646) corresponding to residues 217–646, and nGAP(150–646) corresponding to residues 150–646.

2.4. Yeast heat shock sensitivity assay

S. cerevisiae strains used for the assay were KT27-2B (*MATa ira2::HIS3 ura3 leu2 trp1 his3*) [14] and TK161-R2V (*MATa ura3 leu2 trp1 his3 ade8 RAS2^{Val19}*) [22]. Heat shock sensitivity was determined as described previously [13]. Yeast strains were transformed with various expression plasmids containing the *URA3* gene and were plated onto synthetic complete-uracil (SC-Ura) plates. Heat shock sensitivity assay was performed by replica plating cells to a plate followed by 15–45 min incubation at 55°C. After heat shock treatment, the plate was incubated for 3–4 days at 30°C and photographed.

2.5. In vitro GAP assay

nGAP cDNA encoding residues 1–651 was ligated in frame with the gene encoding glutathione *S*-transferase (GST) and was expressed under the control of the *GAL1* promoter in yeast *S. cerevisiae*. The recombinant GST-nGAP(1–651) fusion protein was purified from the galactose-induced yeast cell lysates by glutathione-Sepharose beads. Small GTPases were purified from *E. coli.* Filter assays were performed according to the method described previously [23]. The small GTPase (1.5 ng) was incubated with 20 ng of either recombinant Gap1^{m} or GST-nGAP(1–651). After washing, radioactivity remaining on the filters was determined.

3. Results

3.1. Molecular cloning of cDNA encoding human nGAP

A fragment of cDNA, which predicted a protein with significant homology to GRD, was obtained during 5′-RACE screening of a human heart cDNA library using the antisense oligonucleotide primer (5′-CTCCACTGGAATTTGGACTG-GAGT-3′) that corresponds to amino acid residues 501–508 (**Asn-Ser-Ser-Pro-Asn**-Ser-**Ser-Gly**) of human ELL2 RNA polymerase II elongation factor [24]. The PCR fragment was subsequently cloned into pCRII vector and used as a probe to screen a human heart cDNA library and a cDNA containing single large open reading frame was obtained. The cDNA contained highly similar sequences with the ELL2 primer in both nucleotide (5′-CTTCCACTGACATTTGGACTG-GAGT-3′; antisense) and amino acid (**Asn-Ser-Ser-Pro-Asn**-Val-**Ser-Gly**) levels. However, there was no significant sequence homology between the two proteins other than the above indicated amino acid stretches.

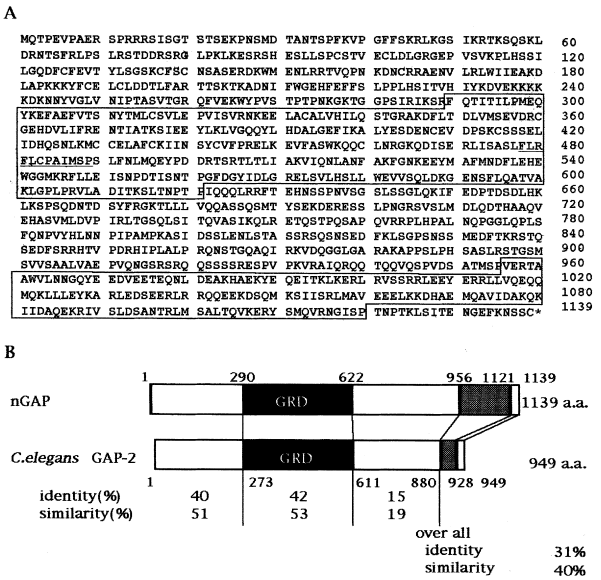

Fig. 1. The amino acid sequence and domain structure of nGAP. A: Amino acid sequence of human nGAP deduced from the nucleotide sequence of the cDNA. Putative GRD (residues 290–621), which is predicted from sequence similarity between p120GAP and neurofibromin, and the C-terminal region (residues 956–1120) that is expected to form a coiled-coil structure are boxed, respectively. B: Schematic representation of nGAP and the *C. elegans* RasGAP, GAP-2 [21]. The black and gray boxes respectively represent the GRD and the region that can form a coiled-coil structure.

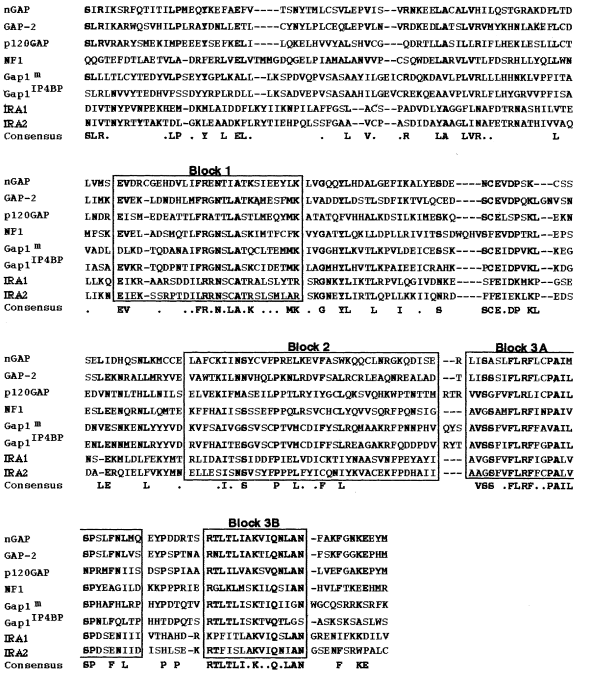

Fig. 2. Multiple sequence alignment of GRDs of known RasGAPs and nGAP. Sequence alignment of GRDs and consensus amino acid sequences are shown. Highly conserved block 1, 2, 3A and 3B are boxed. Source for sequences are *C. elegans* GAP-2 (residues 267–516), p120GAP (residues 705–953), neurofibromin (NF1) (residues 1188–1441), Gap1^{m} (residues 307–561), Gap1^{IP4BP} (residues 280–533), IRA1 (residues 1510–1756), IRA2 (residues 1656–1902) and nGAP (residues 283–530).

The putative open reading frame of the isolated cDNA encodes 1139 amino acids with a calculated molecular mass of 128 570 Da (Fig. 1A). A sequence homology search revealed that the predicted protein exhibits substantial sequence similarity (residues 298–621) to the catalytic domain of all previously reported RasGAPs known as GRD (Fig. 2). Furthermore, amino acid stretches between residues 124 and 158 and residues 169 and 251 show a weak homology to the Pleckstrin-homology (PH) domain and the calcium/phospholipid-binding (CALB) domain, respectively. Notably, p120GAP, Gap1^{m} and Gap1^{IP4BP} possess both the PH domain and the CALB domain. Although the sequence similarity to other RasGAPs is restricted to the GRD domain, the cDNA product exhibits significant homology with a recently identified RasGAP of *C. elegans*, GAP-2 [21], throughout the entire protein sequence, sharing 31% amino acid sequence

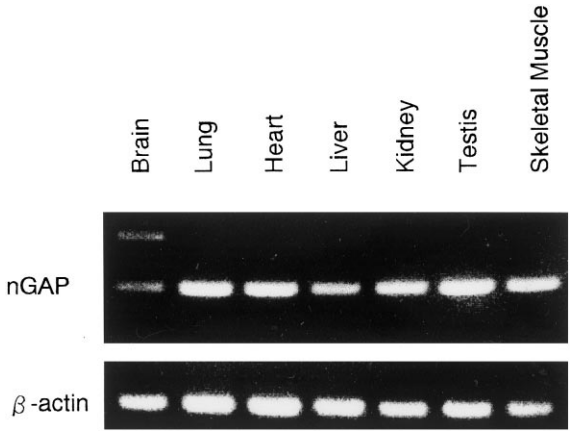

Fig. 3. Tissue distribution of nGAP expression. Expression of nGAP mRNA in various mouse tissues was examined by RT-PCR. Expected size amplified from mouse nGAP mRNA was 286 bp (top row) and the size of genomic DNA amplified with these primers was about 1.7 kb (data not shown). Expression of mouse β-actin mRNA was examined as a control (bottom row).

identity and 40% similarity (Fig. 1B). Furthermore, the two proteins commonly possess a predicted coiled-coil structure at the C-terminal region. Thus, the cloned cDNA encodes a new RasGAP-like protein that appears to be a human homologue of the *C. elegans* GAP-2 and hence we designated the protein as nGAP (where n stands for nematode).

3.2. Tissue distribution of nGAP expression

We next determined the tissue distribution of nGAP expression. On a human tissue RNA blot analysis, we detected 8 kb nGAP mRNA although the signal was very low in many tissues (data not shown). Thus, to analyze expression with greater sensitivity, we performed RT-PCR analysis using mouse tissue mRNA. To do so, a partial cDNA sequence information for mouse nGAP (dbEST clone AA107246) was obtained by searching an EST database. The reported mouse sequence encoded a peptide consisting of 147 amino acids showing 100% homology and 99% identity to the residues 240–386 of human nGAP. Using the mouse sequence, oligonucleotide primers were designed and RT-PCR was performed with poly(A)$^+$ RNA obtained from various mouse tissues (Fig. 3). The result indicated that nGAP mRNA is ubiquitously expressed in adult mouse tissues. Furthermore, RNA derived from brain gave an additional amplified band. This suggests the existence of a differentially spliced form of mRNA or, alternatively, a presence of mRNA that is highly related to nGAP mRNA in brain tissue.

3.3. Effect of nGAP expression on heat shock sensitivity of *ira2*$^-$ or *Ras2*^{Val19} yeast strain

The RasGAP activity of nGAP was next examined by employing a yeast genetic approach. Yeast *S. cerevisiae* possesses two RasGAPs termed Ira1 and Ira2 [15,16]. Genetic disruption of *IRA1* or *IRA2* genes increases intracellular levels of cAMP and, as a result, cells cannot survive following heat shock treatment. However, ectopic expression of mammalian RasGAP in *ira*$^-$ yeast mutants suppresses the elevated Ras activity and the mutant cells survive after heat shock treatment [16–18].

A genetic rescue experiment was performed in order to determine whether human nGAP cDNA would alleviate the heat shock sensitivity of an *ira2*$^-$ yeast strain. As demonstrated in Fig. 4A, full-length nGAP efficiently complemented the *ira2*$^-$ mutation with regard to the heat shock resistance. Next, the nGAP region required for this Ira complementation was examined by using a series of deletion derivatives. The smallest region that could rescue *ira2*$^-$ strain was a fragment encompassing residues 150 and 646 (Fig. 4A). This fragment covered the entire GRD of nGAP (residues 290–621). However, further deletions totally lost the ability to complement yeast Ira2 function in heat shock resistance (data not shown).

The above observation suggested that nGAP functions as a RasGAP and down-regulates yeast Ras activity by stimulating GTP hydrolysis. To further pursue this, we examined the effect of nGAP on constitutively active Ras. A yeast strain TK161-R2V contains Ras2^{Val19}, which has reduced GTPase activity and is resistant to RasGAP. As a result, the yeast strain shows a heat shock sensitive phenotype that is very similar to that of Ira-deficient strains [22]. If nGAP suppressed the heat shock sensitivity of *ira2*$^-$ yeast cells via mechanisms independent of GTP hydrolysis, then the protein would also be expected to suppress the heat shock sensitive phenotype of Ras2^{Val19} cells. On the other hand, if nGAP rescued the *ira2*$^-$ phenotype by stimulating GTP hydrolysis of wild-type Ras, then it should not suppress Ras2^{Val19} phenotype because Ras2^{Val19} is resistant to RasGAP. We found that nGAP did not rescue the Ras2^{Val19} phenotype upon heat shock treatment of TK161-R2V cells (Fig. 4B). Similarly, wild-type neurofibromin-GRD failed to rescue TK161-R2V heat shock sensitivity. In contrast, a Phe^{1434} to Leu mutant of neurofibromin-GRD, NF201, suppressed TK161-R2V heat shock sensitivity because the mutant molecule interacted with the effector region of Ras, which is thought to interact with the downstream Ras effectors, much stronger than the wild-

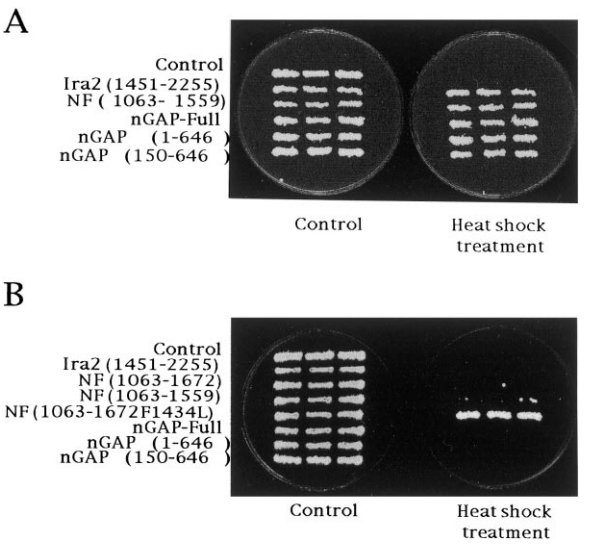

Fig. 4. Heat shock sensitivity assay with *ira2*$^-$ yeast cells. A: Yeast transformants derived from *ira2*$^-$ strain that ectopically express Ira2-GRD, neurofibromin (NF1)-GRD, full-length nGAP, nGAP(1–646) or nGAP(150–646) were heat shock-treated and then incubated at 30°C for 4 days. Replica plate that did not receive heat shock treatment was similarly incubated. B: Yeast strain containing a constitutively active RAS2^{Val19} was transformed with various yeast expression vectors for RasGAPs. Three transformants were subjected to heat shock and then incubated at 30°C for 4 days.

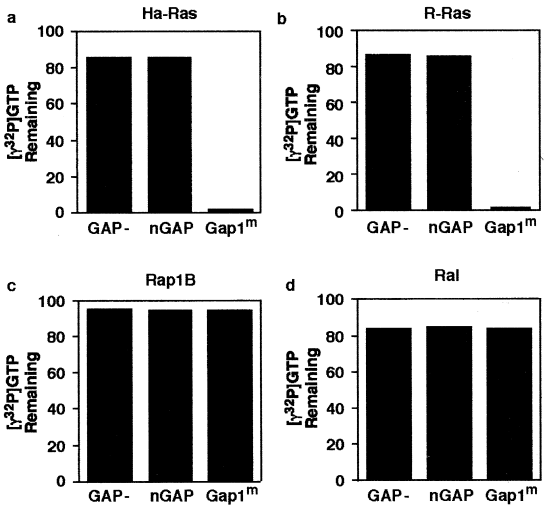

Fig. 5. In vitro GAP assay of nGAP. GAP activity of GST-nGAP(1–651) or Gap1^{m} was examined with the use of [γ-^{32}P]GTP-loaded H-Ras, R-Ras, Rap1B or Ral as a substrate (17 ng per assay). Radioactivity remaining on the filters after 10 min was determined. The amount of [γ-^{32}P]GTP-loaded substrates of samples kept on ice was taken as 100%.

type neurofibromin-GRD [25]. From these observations, we concluded that nGAP is capable of functioning as a RasGAP.

3.4. In vitro GAP assay of nGAP

To directly measure the RasGAP activity of nGAP, we performed in vitro GAP assay with the use of a fusion protein that contains nGAP (amino acid residues 1–651) linked to glutathione *S*-transferase (GST-nGAP). The GST-nGAP fusion protein was found to be biologically active in rescuing heat shock sensitivity when expressed in *ira2*$^{-}$ yeast strain (data not shown). Purification of the fusion protein was confirmed by SDS-polyacrylamide gel electrophoresis and subsequent gel staining (data not shown). GAP activity of GST-nGAP was tested using [γ-^{32}P]GTP-loaded H-Ras, R-Ras, Rap1 or Ral protein prepared in bacteria as substrates. GTP hydrolysis was monitored by using a filter binding assay. Control experiments employing Gap1^{m} exhibited GTP hydrolysis on H-Ras and R-Ras but not on Rap1B and Ral as described previously [23]. In contrast, nGAP failed to exhibit significant GAP activity toward any of the examined low molecular weight GTPases under the conditions tested (Fig. 5).

3.5. Chromosomal mapping

Chromosomal localization of the nGAP gene was determined by fluorescence in situ hybridization (FISH) on normal human metaphase chromosomes using full-length nGAP cDNA as a probe. As shown in Fig. 6, clear discrete signals on both homologues were observed in chromosome 1q25, the region where linkage of a major susceptibility locus to prostate cancer has been recently reported [26,27].

4. Discussion

In this work, we describe the molecular cloning of a human cDNA whose product possesses the GAP-related domain (GRD) that is strongly conserved among all known RasGAPs. The GRD is considered to be indispensable for RasGAPs to activate Ras GTPase activity. Mutational studies of GRD revealed the invariable conservation of several critical amino acids that are needed for Ras binding and for activating Ras GTPase activity [28–31]. In particular, Phe-Leu-Arg-Phe-x-x-Pro-Ala-Ile-x-x-Pro (where x is any amino acid) is the most conserved GRD motif and is present in the nGAP-GRD. Furthermore, structure-function analyses have revealed that three amino acids (Arg^{786}, Lys^{831}, and Arg^{925} that correspond to Arg^{789}, Lys^{834}, and Arg^{928} in human p120GAP) in GRD are required for binding of p120GAP to GTP-bound Ras [31]. In addition, mutation of Lys^{1423} in neurofibromin to Glu or Gln that was detected in patients with colon cancer and anaplastic astrocytoma [32] was suggested to affect the catalytic activity rather than the binding of Ras to neurofibromin. All of these functionally critical amino acids are conserved in GRD of human nGAP (Arg^{369}, Lys^{414}, Arg^{505}, Lys^{512}).

The predicted amino acid sequence strongly suggests that the newly isolated nGAP is a member of RasGAPs. Indeed, expression of nGAP in yeast demonstrated that the protein was capable of rescuing a heat shock sensitive phenotype associated with the lack of yeast RasGAP. Consistently, loss of GAP-2, the *C. elegans* homologue of nGAP, suppressed the larval lethality caused by reduced LET-60 Ras activity [21], indicating that GAP-2 is capable of functioning as a RasGAP in vivo and negatively regulates the LET-60 Ras signalling pathway. Despite extensive effort, however, highly purified recombinant nGAP proteins did not show any in vitro GAP activity toward several low molecular weight GTPase proteins such as H-Ras, R-Ras, Rap1B and Ral. The result suggests that nGAP specifically targets other members of Ras family proteins or low molecular weight GTPases that contain effector domains highly similar to Ras [33–35].

Finally, *nGAP* gene was assigned to the human chromo-

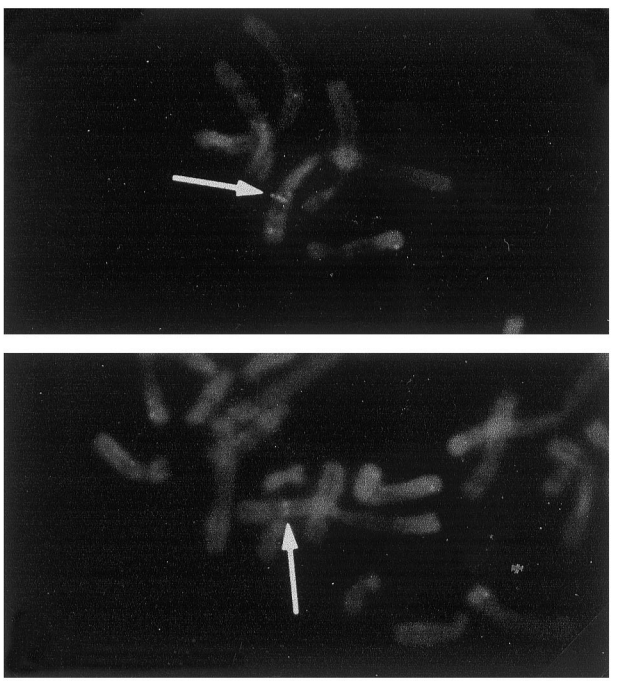

Fig. 6. Fluorescence in situ hybridization mapping of the nGAP gene. A full-length human nGAP cDNA was hybridized to normal human chromosomes as described previously [33]. A metaphase chromosome spread from a single cell counterstained with DAPI and showing nGAP signal on the chromosome 1q25 (arrows).

some 1q25 by FISH. Furthermore, gene map analysis of human genome with the resources of the National Center for Biotechnology Information revealed that *nGAP* gene (human sequence tagged site (STS) WI-30983 that contains 314 bp 3′ non-coding region of *nGAP*) is mapped to the locus between markers D1S218 and D1S466 on chromosome 1q24-25. Intriguingly, the chromosome locus has been recently proposed to contain a putative hereditary prostate cancer susceptible gene (*HPC1*) by genome-wide linkage analysis [26,27]. Since *nGAP* appears to encode a RasGAP as is the case of *NF1* tumor suppressor gene, the chromosomal assignment raises an intriguing possibility that *nGAP* is the proposed *HPC1* gene whose malfunctioning is involved in the development of prostate cancer.

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