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RESEARCH COMMUNICATION

A mutation in the *RET* proto-oncogene in Hirschsprung's disease affects the tyrosine kinase activity associated with multiple endocrine neoplasia type 2A and 2B

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We demonstrate that a Hirschsprung (HSCR) mutation in the tyrosine kinase domain of the *RET* proto-oncogene abolishes in *cis* the tyrosine-phosphorylation associated with the activating mutation in multiple endocrine neoplasia type 2A (MEN2A) in transiently transfected Cos cells. Yet the double mutant RET2AHS retains the ability to form stable dimers, thus dissociating the dimerization from the phosphorylation potential. Co-transfection experiments with single and double mutants carrying plasmids *RET*2A and *RET*2AHS in different ratios drastically reduced the phosphorylation levels of the RET2A protein, suggesting a dominant-negative effect of the HSCR mutation. Also, the phosphorylation associated with the multiple endocrine neoplasia type 2B (MEN2B) allele was affected in experiments with single and double mutants carrying plasmids co-transfected under the same conditions. Finally, analysis of the enzymic activity of MEN2A and MEN2B tumours confirmed the relative levels of tyrosine phosphorylation observed in Cos cells, indicating that this condition, *in vivo*, may account for the RET transforming potential.

INTRODUCTION

The *RET* proto-oncogene encodes an orphan receptor endowed with tyrosine kinase activity [1,2], which has been implicated in the pathogenesis of three dominantly inherited familial cancer syndromes: multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma [3,4,5]. In fact, the molecular defect in all three diseases is represented by point mutations affecting different regions of the *RET* gene. In most MEN2A and familial medullary thyroid carcinoma cases the mutations result in the substitution of one of the cysteine residues within the extracellular domain of RET [3,4]. In MEN2B, a T-to-C transition, within the tyrosine kinase domain, causes the substitution of a Thr residue for a Met residue, at position 918 [5]. Recently, *RET* has been implicated in another neurocristopathy, autosomal dominant Hirschsprung's disease (HSCR) or aganglionic megacolon [6,7]. This disease has been associated with various mutations along the entire *RET* proto-oncogene that produce premature stop codons, alterations of splicing sites or mutated gene products.

In MEN2A and MEN2B, the constitutive activation of RET kinase causes the mutated *RET* alleles to function as dominant transforming genes [8,9]. In MEN2A, the activation seems to result from a ligand-independent dimerization, while in MEN2B it seems to stem from a change in the kinase substrate specificity [10]. HSCR has been attributed to a loss of RET function [11,12].

To investigate whether the HSCR mutation studied exerts a 'loss of function' effect on the activating MEN2A and MEN2B mutations, we analysed the phosphorylation levels of single and double mutants after transfection into Cos cells. We show that the *RET*HSCR mutation predominates over *RET*2A and *RET*2B mutations and also that, in the case of *RET*2A, it has a dominant-negative effect. Moreover, we show that the *RET*2AHS double mutant retains its ability to form stable homodimers, thereby dissociating phosphorylation from dimerization.

We found that the RET phosphorylation in MEN2A and MEN2B tumour samples is similar to that observed in our model system. Hence, Cos cells can be used reliably to study the functional activities of *RET* mutants associated with disease.

EXPERIMENTAL

Plasmid constructions

PSG5-based expression vectors were used as recipient plasmids to clone the full-length proto-*RET* cDNA [8] as an *Eco*R1–*Hin*dIII fragment. Total RNA extracted from MEN2A and MEN2B tumours was reverse-transcribed and subsequently amplified via PCR, using *RET*-specific oligonucleotides encompassing the previously determined mutations [13] at positions 634 (Cys-Arg) and 918 (Met-Thr). The amplified bands carrying the mutations were purified and cloned into the proto-*RET* cDNA, as an *Nde*I–*Bcl*I fragment, replacing the corresponding wild-type segment. The HSCR mutation (Arg-Gln) was obtained by recombinant PCR using two complementary oligonucleotides bearing the desired mutation (CGA-CAA) at codon position 897 as primers [14]. The amplified fragment was isolated and cloned by replacement in the proto-*RET* cDNA. To produce the *RET*2AHS double mutant, the pSG5 vector, carrying the *RET*2A cDNA, was digested with *Eco*R1, and the band containing the mutation was isolated and subsequently cloned into the *Eco*R1 site of the pSG5 vector containing the HSCR mutation (*RET*HSCR) from which the *Eco*R1 fragment containing the wild-type sequence had been removed. For the *RET*2BHS double

Abbreviations used: HSCR, Hirschsprung's disease; MEN2A and MEN2B, multiple endocrine neoplasia type 2A and 2B respectively; anti-P-Tyr: anti-phospho-tyrosine.

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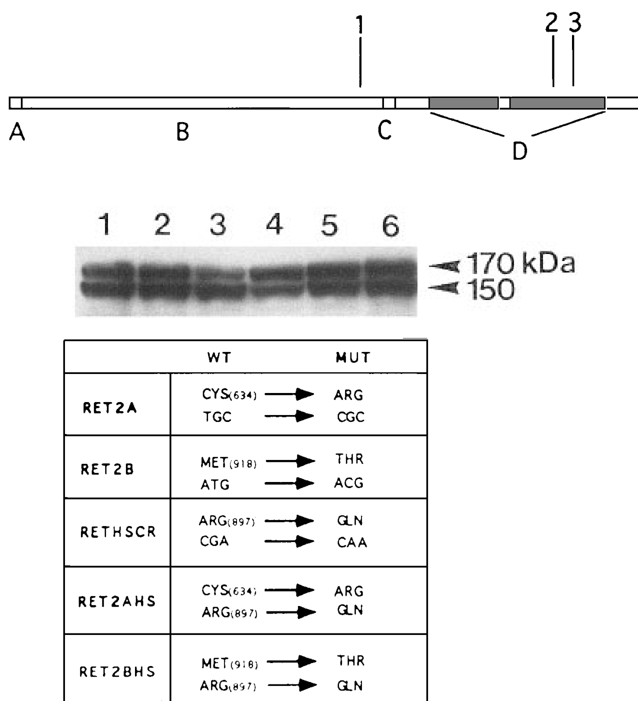

	WT	MUT
RET2A	CYS(634) → TGC →	ARG CGC
RET2B	MET(918) → ATG →	THR ACG
RETHSCR	ARG(897) → CGA →	GLN CAA
RET2AHS	CYS(634) → ARG(897) →	ARG GLN
RET2BHS	MET(918) → ARG(897) →	THR GLN

Figure 1 Analysis of RET proteins from transfected Cos cells

Western blot analysis was carried out on total proteins from cells transfected with the expression vectors corresponding to proto-*RET*, *RET*2A, *RET*2B, *RET*HSCR, *RET*2AHS and *RET*2BHS respectively (lanes 1 to 6), using an anti-RET serum. Proteins were detected by chemiluminescence. The two RET forms and their molecular masses are indicated. A schematic diagram of RET protein is illustrated in the upper part of the Figure: A, B, C and D correspond to the signal peptide, the extracellular, the transmembrane and the tyrosine-kinase domains respectively and 1, 2 and 3 indicate the positions of the mutations investigated. In the lower part of the Figure are reported the nucleotide and amino acid changes associated with MEN2A, MEN2B, HSCR and the double mutants *RET*2AHS and *RET*2BHS respectively.

mutant, the pSG5*RET*HSCR vector was subjected to PCR, using the oligonucleotides 2BFMT (5′AGG ATC CAA AGT GGG AAT3′), containing the MEN2B mutation, and R12S (5′GAT ATG ATC AAA AAG GGA TTC AAT TGC CGT CCA TT3′), which covers *RET* exon 12, as primers. The amplified product was then digested with *Bgl*II and *Bcl*I and the band obtained was cloned into the pSG5 vector containing the proto-*RET* cDNA, previously digested with the same enzymes. All mutant plasmids were sequenced on both strands to confirm the presence of the desired mutations; their positions in the RET protein are illustrated in Figure 1.

Cell cultures and transfection

Cos and CV-1 cells were grown as described previously [15]. Transfection was carried out by the calcium coprecipitation technique [16]. Forty-eight hours after transfection, cells were lysed in JS buffer consisting of 66 mM Hepes (pH 7.4), 200 mM NaCl, 1% (v/v) glycerol, 1% (w/v) Triton X-100, 2 mM $MgCl_2$, 6 mM EGTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 2 mM PMSF. Protein concentration was determined by a modified Bradford method (Bio-Rad) [17]. Equal amounts of proteins were separated by SDS/7.5%-PAGE, transferred onto a membrane and subsequently hybridized with an antibody directed against a peptide from the C-terminus of the RET protein (a gift from Dr. M. Santoro, Centro di Endocrinologia e Oncologia Sperimentale de CNR, Naples, Italy) or an anti-phosphotyrosine (anti-P-Tyr) monoclonal antibody (ICN Biochemicals, Cleveland, OH, U.S.A.). Detection was achieved by chemiluminescence, using the ECL kit (Amersham, Buckinghamshire, U.K.). To detect RET dimers, electrophoresis was carried out with SDS/PAGE under non-reducing conditions. To analyse the RET protein from MEN2A and MEN2B tumours, the tissues were homogenized in JS buffer and processed as described above.

RESULTS AND DISCUSSION

To study the functional activities of the various forms of RET, transient transfection experiments were performed with expression vectors carrying the cDNAs corresponding to proto-*RET*, *RET*2A, *RET*2B and *RET*HSCR mutants. To analyse the effect of the HSCR mutation on the activating *RET*2A and *RET*2B mutations, double mutants, *RET*2AHS and *RET*2BHS respectively, were generated by introducing the HSCR mutation in the cDNAs corresponding to MEN2A and MEN2B alleles. The enzymic activity of all products was analysed *in vivo* after transfection into recipient Cos cells, which have negligible levels of endogenous RET and allow high levels of protein production from the transfected cDNAs under the transcriptional control of the SV40 enhancer/promoter region [15]. Protein extracts prepared 48 h after transfection with the various pSG5 constructs were analysed by Western blot. Two molecular species of 170 and 150 kDa, corresponding to the mature and immature glycosylated forms respectively, were identified with an anti-RET serum [18] (Figure 1).

Phosphorylation on tyrosine residues was then examined to evaluate the levels of phosphorylation *in vivo* of the various RET proteins. Equal amounts of proteins from transfected cells were loaded on twin gels, transferred onto a membrane and immunoblotted with anti-RET serum or anti-P-Tyr monoclonal antibody respectively. RET was detected at variable levels for each transfectant (Figure 2A). Interestingly, RETHSCR and the double mutants RET2AHS and RET2BHS displayed no tyrosine-phosphorylation (Figure 2B, lanes 4, 5 and 6). In contrast, RET2A and RET2B mutants were highly phosphorylated (Figure 2B, lanes 2 and 3). This result suggests that the HSCR pre-

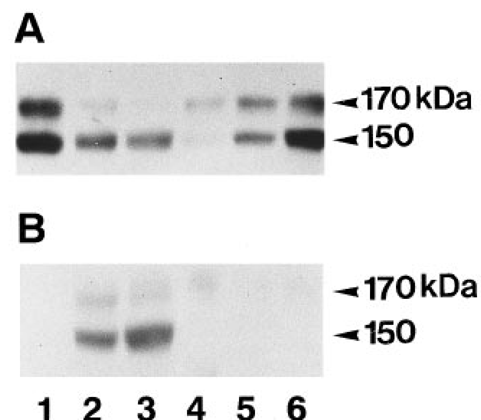

Figure 2 Tyrosine phosphorylation of RET proteins in vivo

Cos cells transfected with the expression vectors containing the cDNAs coding for proto-*RET*, *RET*2A, RET2B, *RET*HSCR, *RET*2AHS and *RET*2BHS (lanes 1 to 6) were lysed in JS buffer in the presence of sodium orthovanadate as phosphatase inhibitor. Western blot analysis was performed on comparable amounts of proteins challenged with an anti-RET serum (**A**) or an anti-P-Tyr monoclonal antibody (**B**). Detection was achieved by chemiluminescence. The sizes of the RET forms are indicated.

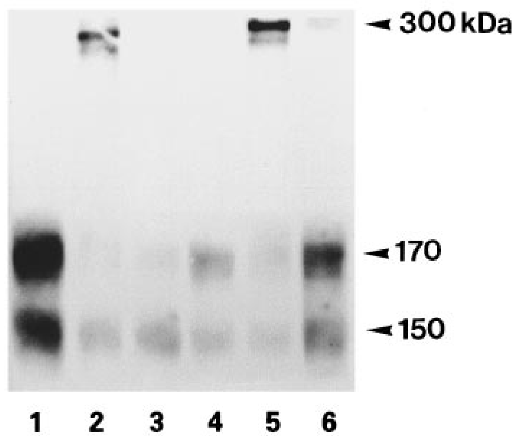

Figure 3 Dimerization analysis of proto-RET and its mutants

Comparable amounts of proteins from cells transfected with the various *RET* constructs were fractionated by SDS/PAGE under non-reducing conditions and, after transfer, reacted with an anti-RET serum and detected by chemiluminescence. Lanes 1–6 correspond to proto-RET, RET2A, RET2B, RETHSCR, RET2AHS and RET2BHS proteins respectively. The arrows indicate the 150 and 70 kDa RET monomers and the approx. 300 kDa RET dimer.

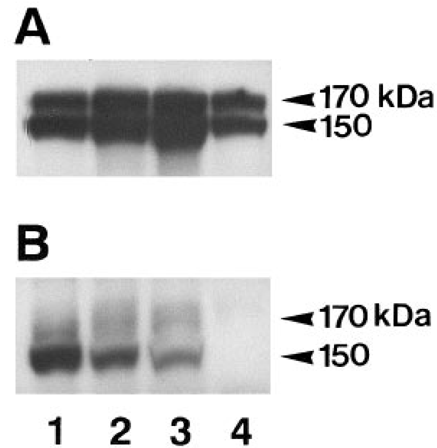

Figure 4 Dominant negative effect of the HSCR mutation over MEN2A

Cos cells were co-transfected with 10 μg of the expression vectors containing the cDNAs coding for *RET*2A and 10 or 30 μg of *RET*2AHS. Parallel transfections were carried out with 10 μg of *RET*2A and *RET*2AHS expression vectors alone. Cells were lysed in JS buffer in the presence of sodium orthovanadate as phosphatase inhibitor. Western blot analysis was performed on increasing amounts of proteins: lane 1, RET2A (50 μg); lane 2, RET2A and RET2AHS at a ratio of 1:1 (100 μg); lane 3, RET2A and RET2AHS at a ratio of 1:3 (200 μg); lane 4, RET2AHS (50 μg). After transfer, the proteins were challenged with anti-RET serum (**A**) or an anti-P-Tyr monoclonal antibody (**B**). Detection was achieved by chemiluminescence. The two RET forms and their molecular masses are indicated.

dominates over the MEN2A and MEN2B mutations. Proto-RET, as expected, showed no phosphorylation (Figure 2B, lane 1). The same results were repeatedly obtained in transfections using increasing amounts (1–10 μg) of plasmid DNA: in all cases, RET2A and RET2B were the only phosphorylated forms (results not shown).

It has been proposed that MEN2A mutations convert *RET* into a transforming gene because the mutants can form stable dimers, thus constitutively activating the tyrosine kinase [8,9]. The Cys residues present in the extracellular domain of the protein are involved in disulphide bonds. The MEN2A mutations make the number of such residues uneven; therefore, aberrant intermolecular disulphide bonds can be formed with other RET mutant partners, generating stable homodimers capable of being phosphorylated [8,9]. We examined whether the HSCR mutation could affect the dimer formation and/or the kinase activation of the *RET* variants transfected. Equal amounts of proteins from cells transfected with the various *RET* constructs were separated in non-reducing SDS/PAGE and, after transfer to a membrane, they were challenged with an anti-RET serum. In addition to the 150 and 170 kDa species, which correspond to RET monomers, a band with an apparent molecular mass of 300 kDa, probably corresponding to RET dimers, was detected only in RET2A and in the RET2AHS double mutant (Figure 3, lanes 2 and 5). This result indicates that RET2A is the only RET form able to produce stable dimers and that the *RET*HSCR mutation does not interfere with this capacity.

To test whether the HSCR mutation has a dominant-negative effect, transient co-transfections were performed using a constant amount of the vector expressing the *RET*2A mutant and two different increasing amounts of the vector expressing the *RET*2AHS double mutant in a final proportion of 1:1 and 1:3. In order to normalize the amount of RET2A, proteins were analysed in accordance with the different ratios of DNA used for transfection. Twin polyacrylamide gels were run in parallel and, after separation and transfer, the proteins were immunoblotted with anti-RET serum or anti-P-Tyr monoclonal antibody respectively. Only in the presence of both expression vectors were the bands corresponding to the two forms of RET detectable in increasing amounts (Figure 4A, lanes 2 and 3), while its relative phosphorylation level decreased remarkably (Figure 4B, lanes 2 and 3). RET2A and RET2AHS displayed the expected phosphorylation levels (Figure 4B, lanes 1 and 4). These data suggest that the HSCR mutant acts through a dominant-negative mechanism. The same results were obtained in CV-1 cells, in which no amplification of the transfected plasmids occurs (results not shown).

If the constitutive activation of the tyrosine kinase activity of RET observed in the transfection assay in Cos cells has any relevance *in vivo* in the establishment of tumours associated with MEN2A and MEN2B, mutant *RET* alleles should be expressed in these tumours and comparably phosphorylated on tyrosine residues. To test this hypothesis, the expression of RET and its phosphorylation status was investigated in two MEN2A and two MEN2B medullary thyroid carcinoma tumour tissues harbouring the same mutations tested in Cos cells [13]. Each type of tumour gave the same results; see Figure 5 for an example of this analysis. Equal amounts of the RET proteins were immunoprecipitated with an anti-RET antibody and the two glycosylated forms were identified by Western blot analysis. RET2A was more abundant than RET2B and in both samples the 150 kDa form was more prominent than the 170 kDa form (Figure 5A, lanes 1 and 2). When comparable amounts of RET proteins were first immunoprecipitated with anti-RET antibodies and then challenged with an anti-P-Tyr monoclonal antibody, both forms were phosphorylated (Figure 5B, lanes 1 and 2).

Cos cells have been used widely as a tool to study the functional activities of proteins produced at a high rate after transfection of the corresponding expression vectors [15]. Here we show that Cos cells are a suitable system with which to analyse the tyrosine kinase activity of *RET* gene products. The results were reproduced with a wide range of plasmid concentrations in transfection experiments, ruling out a spurious ligand-independent activation that would invalidate the results obtained with the mutant constructs.

We provide experimental evidence that the HSCR mutation

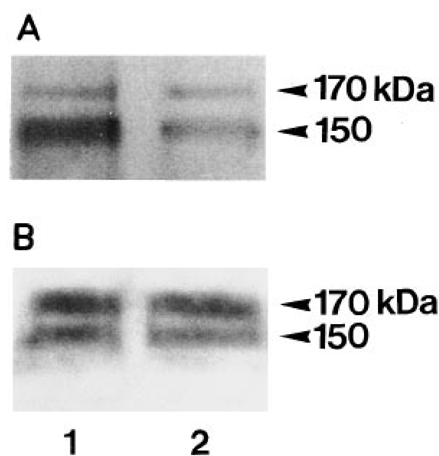

Figure 5 Analysis of RET phosphorylation in MEN2A and MEN2B tumour tissues

Medullary thyroid carcinoma tissues were homogenized in JS buffer with sodium orthovanadate as phosphatase inhibitor. Comparable amounts of RET proteins from a MEN2A (lane 1) and a MEN2B (lane 2) tumour sample were immunoprecipitated with a polyclonal antibody to RET. The immunocomplexes were then analysed by Western blot, using either an anti-RET antibody (**A**) or an anti-P-Tyr monoclonal antibody (**B**) and detected by chemiluminescence. The two RET forms are indicated.

studied, localized in the tyrosine kinase domain of RET protein, shows a phosphorylation level *in vivo* that is indistinguishable from that of proto-RET. HSCR is, in fact, thought to be due to a loss of RET function [11,12]. This is in accordance with the finding that the mutation studied affects an amino acid residue that is highly conserved in at least 90 % of the protein tyrosine kinases.

We also show that this mutation predominates in *cis* over the activating MEN2A mutation, suppressing its phosphorylation potential. This effect is dissociated from the dimerization capability of RET2A. The results support the hypothesis that RET activation in MEN2A is a consequence of a ligand-independent dimerization of the receptor and that the HSCR mutation can overcome it. The 'loss of function' effect of the HSCR mutation on MEN2A is further demonstrated by the dominant-negative effect of the co-transfection experiments, probably due to heterodimerization of the RET2A and RET2AHS proteins and/or competition for downstream substrates. Recently, it has been shown that three different HSCR mutations, all localized in the tyrosine kinase domain of RET, abrogate the biological properties and significantly affect the enzymic activity of *RET*/PTC2, a chimeric transforming version of *RET* [12]. We introduced the HSCR mutation in a more 'physiological' context, i.e. in a complete and correctly processed RET molecule. We were thus able to study the phosphorylation activity in natural RET versions and to detect any impairment of the dimerization potential of the molecule. In fact we provide the first demonstration that the RET2AHS double mutant retains its ability to form stable homodimers while its kinase activity is completely suppressed.

RET activation can be due to a variety of mechanisms in MEN2B. The *RET*2B mutation induces autophosphorylation and, more importantly, can change the peptide substrate specificity. Inappropriate substrates would then be phosphorylated, stimulating pathways normally activated by a different class of tyrosine kinases [10]. Here we show that the MEN2B mutation does indeed cause increased phosphorylation and that the HSCR mutation is predominant, drastically reducing its phosphorylation capacity. The two mutations affect amino acid residues coded for by the same exon and lie in same region of the tyrosine kinase domain, only 21 amino acids apart. The MEN2B mutation could change the substrate specificity by inducing the stretches of amino acids similar to v-src, present in that region [19], to fold into a different structure so as to recognize and interact with different substrates [10]. The HSCR mutation might interfere with this new structure and inhibit its phosphorylation.

The analysis of tumour samples showed that RET phosphorylation levels may be important in the establishment of MEN2A and MEN2B tumours *in vivo*. Both RET2A and RET2B were tyrosine-phosphorylated similarly to the proteins produced in Cos cells. Although the number of tumours examined is too small to draw general conclusions, it is noteworthy that in transfection experiments and in tumours both forms are highly phosphorylated.

In conclusion, the experimental system described here is a rapid and efficient method with which to test the tyrosine phosphorylation and the relevant dominance of the various *RET* mutants

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REFERENCES

1 Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. (1988) Oncogene **3**, 571–578
2 Takahashi, M., Buma, Y. and Hiai, H. (1989) Oncogene **4**, 805–806
3 Mulligan, L. M., Kwok, J. B., Healey, C. S., Elsdon, M. J., Eng, C., Gardner, E., Love, D. R., Mole, S. E. and Moore, J. K. (1993) Nature (London) **363**, 458–460
4 Donis-Keller, H., Dou, S., Chi, D., Carlson, K. M., Toshima, K., Lairmore, T. C., Howe, J. R., Goodfellow, P. and Wells, S. A. (1993) Hum. Mol. Genet. **2**, 851–856
5 Hofstra, R. M. W., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W. M., Ploos van Amstel, H. K., Romeo, G. et al. (1994) Nature (London) **367**, 375–376
6 Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. and Martusciello, G. (1994) Nature (London) **367**, 377–378
7 Edery, P., Lyonnet, S., Mulligan, L. M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Feketé, C., Ponder, B. A. J. and Munnich, A. (1994) Nature (London) **367**, 378–380
8 Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Grieco, M., Fusco, A., Vecchio, G., Matoskova, B., Kraus, M. H. and Di Fiore, P. P. (1995) Science **267**, 381–383
9 Asai, N., Iwashita, T., Matsuyama, M. and Takahashi, M. (1995) Mol. Cell. Biol. **15**, 1613–1619
10 Songyang, Z., Carraway, K. L., III, Eck, M. J., Harrison, S. C., Feldman, R., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C. et al. (1995) Nature (London) **373**, 536–539
11 Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994) Nature (London) **367**, 380–383
12 Pasini, B., Borrello, M. G., Greco, A., Bongarzone, I., Luo, Y., Mondellini, P., Alberti, L., Miranda, C., Arighi, E., Bocciardi, R. et al. (1995) Nature (London) Genetics **10**, 35–40
13 Quadro, L., Panariello, L., Salvatore, D., Carlomagno, F., Del Prete, M., Nunziata, V., Colantuoni, V., Di Giovanni, G., Brandi, M. L., Mannelli, M. et al. (1994) J. Clin. Endocrinol. Metab. **79**, 590–594
14 Higuchi, R. (1989) Using PCR to Engineer DNA. PCR Technology (Erlich, A., ed.), Stockton Press Ltd., Basingstoke
15 Gluzman, Y. (1981) Cell **23**, 175–182
16 Graham, F. L. and Van der Eb, A. J. (1973) Virology **52**, 456–467
17 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
18 Takahashi, M., Asai, N., Iwashita, T., Isomura, T., Miyazaki, K. and Matsuyama, M. (1993) Oncogene **8**, 2925–2929
19 Hanks, S. K., Quinn, A. M. and Hunter, T. (1988) Science **241**, 42–52

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