The EMBO Journal vol.6 no.9 pp.2661-2667, 1987

Phosphorylation of the human transferrin receptor by protein kinase C is not required for endocytosis and recycling in mouse 3T3 cells

Marino Zerial, Maarit Suomalainen, Margherita Zanetti-Schneider[1], Claudio Schneider[1] and Henrik Garoff[2]

Cell Biology Programme, and [1]Differentiation, European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, 6900 Heidelberg, FRG

[2]Present address: Karolinska Institutet, Center for Biotechnology, Department of Molecular Biology K87, Huddinge University Hospital, S-14186 Huddinge, Sweden

Communicated by K.Simons

We have investigated the role of phosphorylation in the endocytosis of the human transferrin receptor (TR) by replacing its phosphorylation site, Ser24, with Ala through site-directed mutagenesis of the TR cDNA. The TR Ala24 mutant expressed in mouse 3T3 cells was not phosphorylated, even following stimulation of protein kinase C by phorbol ester. However, in spite of this defect the mutant was efficiently endocytosed and recycled back to the plasma membrane with kinetics similar to those of TR and a control mutant TR Ala63. Thus, these results confirm earlier results by Davis *et al.* (1986, *J. Biol. Chem.*, 261, 9034-9041) that Ser24 of human TR is the phosphorylation site for protein kinase C but do not support a role of this modification as a signal for TR endocytosis and recycling.

Key words: transferrin receptor/endocytosis/phosphorylation/site-directed mutagenesis

Introduction

Iron uptake in animal cells is accomplished via a process of receptor-mediated endocytosis. Transferrin receptor (TR) binds and internalizes its specific iron-loaded ligand, transferrin (see May and Cuatrecasas, 1985 for a review). The human TR consists of two identical transmembrane glycoproteins of 760 amino acids (Schneider *et al.*, 1984; McClelland *et al.*, 1984) linked by two intermolecular disulfide bonds (Jing and Trowbridge, 1987). The molecular mechanisms responsible for iron delivery are essentially based upon the difference in pH between the external and internal (endosomal) milieu. Ferro-transferrin binds to TR at the neutral pH of blood and the complex is internalized via coated pits and routed to the endosome via coated vesicles (Booth and Wilson, 1981; Pearse, 1982; Harding *et al.*, 1983; Hopkins, 1983; Hopkins and Trowbridge, 1983; Willingham *et al.*, 1984). At the acidic pH in the endosomal compartment, iron dissociates while apo-transferrin remains bound to the receptor and recycles back to the cell surface (Octave *et al.*, 1981; Bleil and Bretscher, 1982; Klausner *et al.*, 1983; Dautry-Varsat *et al.*, 1983; Ciechanover *et al.*, 1983). Here at physiological pH, apo-transferrin finally dissociates from TR. Studies on the kinetics of internalization of transferrin have shown that surface-bound ferro-transferrin is rapidly endocytosed, with an estimated $t_{1/2}$ ranging between 2.5 and 7.5 min (Bleil and Bretscher, 1982; Hopkins and Trowbridge, 1983; Hopkins, 1983; Ciechanover *et al.*, 1983; Klausner *et al.*, 1984; Watts, 1985) and rapidly recycles back to the plasma membrane. The complete cycle occurs in 6-16 min depending on the cell type (in K562 and HepG2 cells respectively; Ciechanover *et al.*, 1983; Klausner *et al.*, 1984).

Although the mechanism operating in iron uptake has been successfully elucidated, very little is known about the mechanisms controlling TR endocytosis and recycling. In particular, it would be interesting to know which molecular features of TR are responsible for clustering into coated pits and which structures are involved in receptor recycling. A very attractive hypothesis is that phosphorylation and dephosphorylation of the TR cytoplasmic tail control internalization and recycling (see a recent review by Sibley *et al.*, 1987). This hypothesis originated from experiments showing a concomitant effect of phorbol esters on both TR phosphorylation and the number of transferrin-binding sites on the cell surface. It has been shown that phorbol esters enhance phosphorylation of TR both *in vitro* and *in vivo* by stimulating protein kinase C (Castagna *et al.*, 1982; May *et al.*, 1984, 1985a,b). This effect is followed *in vivo* by a redirecting of TR from the surface pool to the internal pool: such a decrease in the number of cell surface transferrin-binding sites has been termed down-regulation of the receptor (Klausner *et al.*, 1984; May *et al.*, 1984, 1985a,b). Removal of the protein kinase C activator leads to dephosphorylation and subsequent reappearance of the original amount of transferrin-binding sites at the cell surface (May *et al.*, 1984). These experiments have suggested that the effect of phorbol ester reflects a normally occurring mechanism controlling TR endocytosis and recycling. Phosphorylation could be required for internalization and dephosphorylation for recycling.

In order to evaluate this hypothesis we studied the endocytosis of a phosphorylation-defective human TR mutant expressed in mouse 3T3 cells. For this purpose, we used site-directed mutagenesis of human TR cDNA to replace Ser24 by Ala. This residue of the TR cytoplasmic domain has recently been shown to be the only phosphorylation site for protein kinase C (Davis *et al.*, 1986). We also constructed a control mutant in which Ser63 was converted into Ala. The mutants as well as the wild-type TR cDNAs were then used to stably express the corresponding proteins in mouse 3T3 cells. Endocytosis of the human wild-type and mutant TRs was studied using ^{125}I-labeled Fab fragments prepared from a monoclonal antibody specific for human TR. The results show that phosphorylation of the human TR cytoplasmic domain does not play a role as a signal for endocytosis and recycling in 3T3 cells. Interestingly, upon phorbol ester stimulation of 3T3 and transformed cells, although the phosphorylation state of the endogenous mouse TR as well as the heterologous human TR was increased, TR down-regulation did not occur in these cells.

Results

Construction and in vitro analysis of mutants

A 0.9-kb *Hin*dIII-*Hin*dIII fragment encoding the first 292 N-terminal residues of TR was isolated from pGEM1 TR, a vector containing the entire TR cDNA (Zerial *et al.*, 1986), and inserted

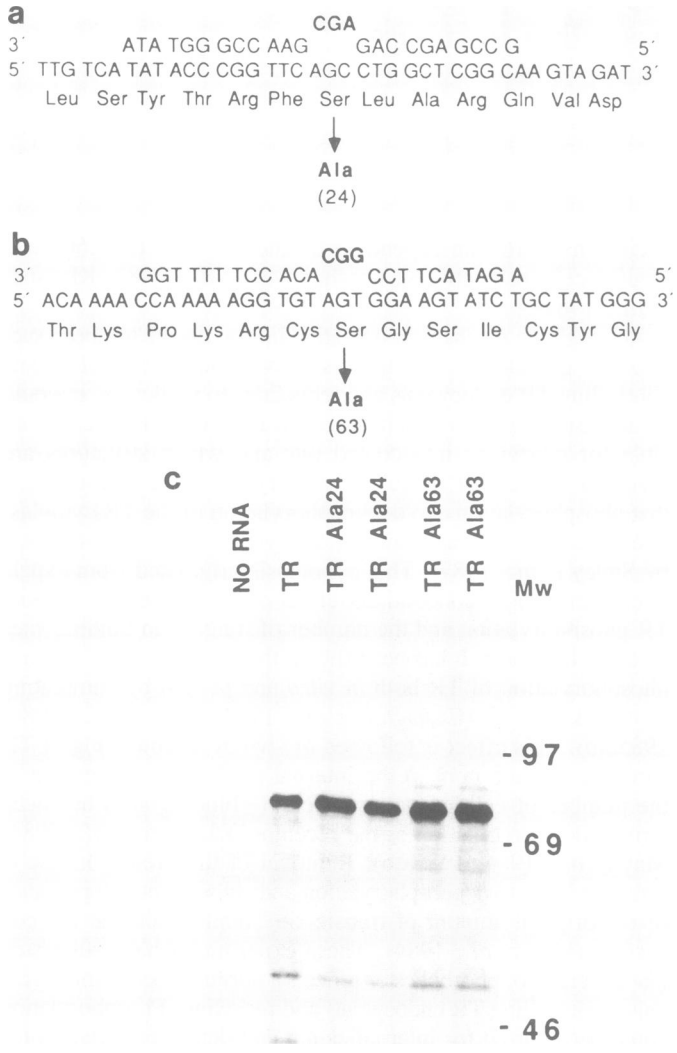

Fig. 1. Construction of human TR mutants. The synthetic oligonucleotides (25 mers) used to construct the TR Ala24 and TR Ala63 mutants are shown in **(a)** and **(b)** respectively, above each complementary sequence [shown from nucleotides 315 to 353 in **(a)** and from 432 to 470 in **(b)**; Schneider *et al.*, 1984] in the human TR cDNA. Below the nucleotide sequences the deduced amino acid sequences are indicated. The three base substitutions (bold letters) convert each serine into alanine. **(c)** Fluorogram of SDS–PAGE analyses of [^{35}S]methionine-labeled proteins obtained by translating pGEM1 TR, pGEM1 TR Ala24 and pGEM1 TR Ala63 mRNAs in a rabbit reticulocyte lysate. The correct engineering of pGEM1 TR Ala24 and pGEM1 TR Ala63 was controlled by comparison of their *in vitro* transcription–translation products with that of pGEM1 TR, as previously described (Zerial *et al.*, 1986). A translation performed in the absence of TR-specific mRNA is included as a control. The mol. wt markers are phosphorylase B (97kd), albumin (69kd) and ovalbumin (46kd).

into M13 mp19. Mutagenesis was carried out by primer extension on the single-stranded phage DNA using the oligonucleotides shown in Figure 1a and b. Colony hybridization and DNA sequencing were used for the selection of mutants. Next, small, completely sequenced DNA fragments (*Xho*I–*Dde*I, 324 bp) containing the mutations Ala24 (the phosphorylation mutant) and Ala63 (the control mutant) respectively were inserted into pGEM1 TR to yield pGEM1 TR Ala24 and pGEM1 TR Ala63. The pGEM1 constructions were then used for *in vitro* transcription–translation studies to check for correct engineering of mutant cDNA (maintenance of reading frame). Figure 1c shows that this is indeed the case: the mutant TRs have the same electrophoretic mobility as the wild-type TR (Zerial *et al.*, 1986).

Expression of wild-type and mutant human TRs in mouse cells

To obtain expression in animal cells the wild-type and the two mutant TR cDNAs were inserted into the expression vector pSVd 2-3 tss$^-$ (containing the SV40 early promoter; D.Huylebroeck *et al.*, in preparation).

Mouse 3T3 cells were co-transfected with pSV Neo (Colbere-Garapin *et al.*, 1981) as a selectable marker and either pSVTR, pSVTR Ala24 or pSVTR Ala63. Colonies of cells resistant to the antibiotic G418 were isolated. Clones expressing human wild-type and mutant TRs were selected through immunofluorescence staining using the mouse monoclonal anti-human TR antibody (OKT9) followed by rhodamine-conjugated goat anti-mouse immunoglobulin. HeLa and untransfected 3T3 cells were used as positive and negative controls respectively. About 100 positive transformants/1 $\times$ 10^6 cells were obtained for TR, TR Ala63 and TR Ala24. One of each group (called TR/1, TR Ala63/2 and TR Ala24/5) was chosen for further studies.

For the endocytosis studies it was essential to establish that the human TR and TR mutants were expressed on the cell surface as transferrin-binding homodimers, as they normally are in human cells. In this respect, a major concern of ours was the possibility of formation of heterodimers between murine and human TR chains as previously documented by Newman *et al.* (1983). The presence of these molecules would make interpreting the results on the endocytosis of mutant TRs very difficult.

In order to determine whether hybrid receptors are formed in our clones, we sequentially immunoprecipitated human and mouse TRs (and vice versa) from lysates of TR/1, TR Ala63/2 and TR Ala24/5 cells metabolically labeled with [^{35}S]methionine. First, essentially all the human TR was immunoprecipitated from the cell lysate through two incubations with the mouse monoclonal anti-human TR antibody PA-1 (Moldenhauer, unpublished, see Materials and methods). Second, the mouse TR was immunoprecipitated from the same lysate by the rat monoclonal anti-mouse TR antibody H.129.121 (Van Agthoven *et al.*, 1984). The experiment was also performed with the opposite order of immunoprecipitation (data not shown).

Figure 2 shows the analysis of the immunoprecipitated material by SDS–PAGE under reducing (upper panel) and non-reducing conditions (lower panel).

Analysis of HeLa and 3T3 control cells shows that the monoclonal antibodies PA-1 and H.129.121 can efficiently and specifically immunoprecipitate the human TR from HeLa cells (see lanes 1 and 2) and the mouse TR from 3T3 cells (lanes 3 and 4) respectively. The two receptors can be distinguished in the reduced form because of a difference in their electrophoretic mobility: the human TR migrates as a single band at ~90 kd (see HeLa, lanes 1 and 2) whereas the mouse TR forms a slightly slower migrating double band (see 3T3, lane 3). The heterogeneity of mouse TR has been previously noted and attributed to differences in the carbohydrate residues (van Agthoven *et al.*, 1984).

Analysis of the transformed cells (upper panel Figure 2) shows that the two sequential treatments with anti-human TR antibody deplete the lysate of human TR but not of mouse TR. Mouse TR was subsequently immunoprecipitated with the anti-mouse TR antibody. Analogous results were obtained when the immunoprecipitation was performed in the opposite order (data not shown). The reduced monomeric human TR immunoprecipitated from the transformed clones seems to be somewhat larger than in HeLa cells (compare lanes 1 and 2 in HeLa, TR/1, TR Ala

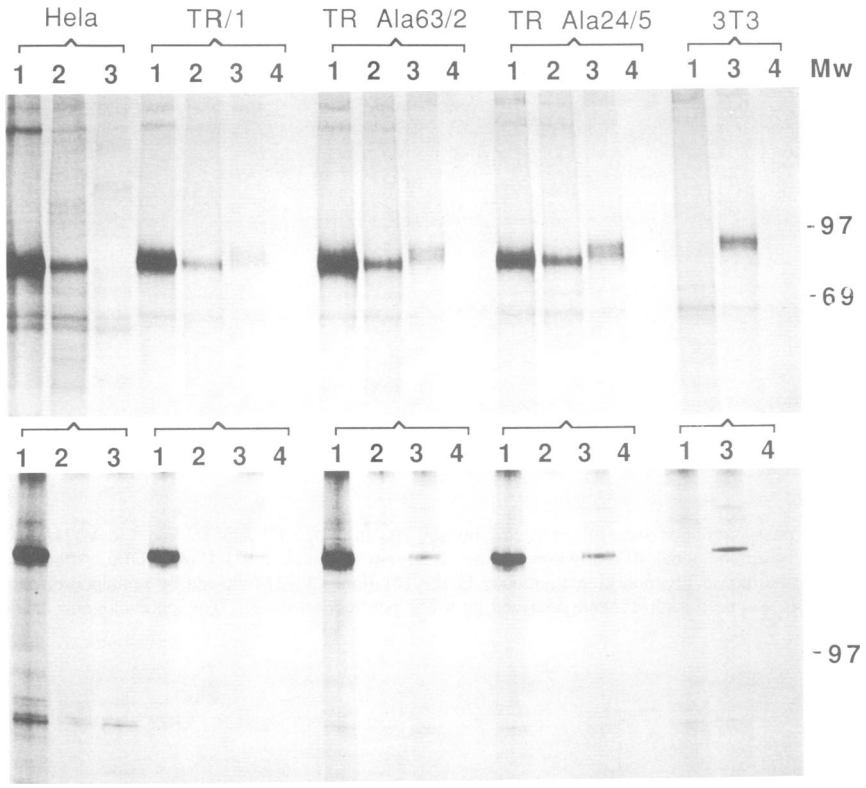

Fig. 2. Fluorogram of human wild-type and mutant TRs and mouse TR isolated by immunoprecipitation with the monoclonal antibodies PA-1 (anti-human TR) and H.129.121 (anti-mouse TR), respectively. HeLa, TR/1, TR Ala63/2, TR Ala24/5 and untransfected 3T3 cells were metabolically labeled with [^{35}S]methionine. Human TR was first immunoprecipitated from the cell lysates through two incubations with the monoclonal antibody PA-1 **(lanes 1** and **2)**; this procedure was followed by two rounds of immunoprecipitation of the mouse TR using the monoclonal antibody H.129.121 **(lanes 3** and **4)** (see Materials and methods). Reduced (upper panel) and unreduced (lower panel) samples were analyzed by SDS–polyacrylamide gel electrophoresis on 8 and 6% polyacrylamide gels respectively. The fluorograms were exposed for 3 days. Note that HeLa and 3T3 cell lysates were incubated only once with anti-mouse and anti-human TR monoclonal antibodies, respectively.

63/2 and TR Ala 24/5 cells), but it can still be distinguished from the larger mouse TR (compare lanes 3 and 4 in TR/1, TR Ala 63/2, TR Ala 24/5 and untransfected 3T3 cells). Under non-reducing conditions (lower panel), 180-kd dimers but no 90-kd monomers can be seen in all samples. We conclude that the large majority of the human wild-type and mutant TR chains are expressed as homodimers in 3T3 cells. A small amount of hybrid TR might still be present, but this should be of no significance for our experiments. Note that the human TR is expressed in much higher amounts than the endogenous receptor. Quantification of bands showed that the human TR is expressed 16, 10 and 5 times higher than the endogenous mouse TR in TR/1, TR Ala63/2 and TR Ala24/5 cells respectively. This high expression level has facilitated our analyses considerably.

The expression of TR homodimers on the cell surface was determined by immunofluorescence (not shown), and quantitative data were obtained by using transferrin and Fab fragment (of PA-1 antibody) binding (see below). All assays showed that the human TR is present on the plasma membrane of TR/1, TR Ala63/2 and TR Ala24/5 cells. The binding studies (see below) suggest that ~25% of human TR is present on the cell surface. From these experiments we conclude that the system fulfils the necessary prerequisites for studying receptor endocytosis.

TR Ala24 cannot be phosphorylated

To confirm that Ser24 is indeed the phosphorylation site on TR we labeled the cells in the presence of [^{32}P]phosphate. Normally, the level of phosphorylation is quite low and sometimes difficult to detect, but it can be increased several times by phorbol esters (May *et al.*, 1984, 1985a; Davis *et al.*, 1986). We therefore determined the phosphorylation state of TR in TR/1, TR Ala63/2 and TR Ala24/5 cells, when stimulated with 100 nM 4-β-phorbol-12,13-dibutyrate (PDBu) for 40 min in medium containing [^{32}P]phosphate.

Figure 3 shows the results of the immunoprecipitated mouse (lanes 1–2) and human (lanes 3–4) TRs from unstimulated (lanes 1 and 3) and stimulated (lanes 2 and 4) cells. Human and mouse TRs are very weakly detected in unstimulated cells (seen on an overexposure of the gel of Figure 3, not shown) as expected from earlier experiments (May *et al.*, 1984; Davis *et al.*, 1986).

Addition of phorbol ester strongly enhances the phosphorylation state of human TR and TR Ala63. The phosphorylation of the mouse TR is also clearly increased. On the contrary, TR Ala24 cannot be phosphorylated even in stimulated cells (compare lanes 3–4 of TR/1, TR Ala63/2 with TR Ala24/5). These results show that the mutant TR Ala24 cannot be phosphorylated *in vivo* upon stimulation of protein kinase C and confirm the finding by Davis *et al.* (1986) that Ser24 is the major phosphorylation site in the human TR molecule.

TR Ala24 is normally endocytosed and recycled

Initially we planned to use ^{125}I-labeled human ferro-transferrin to study TR internalization and recycling (Klausner *et al.*, 1983; Dautry-Varsat *et al.*, 1983; Ciechanover *et al.*, 1983). However, the human transferrin was also found to bind to the mouse recep-

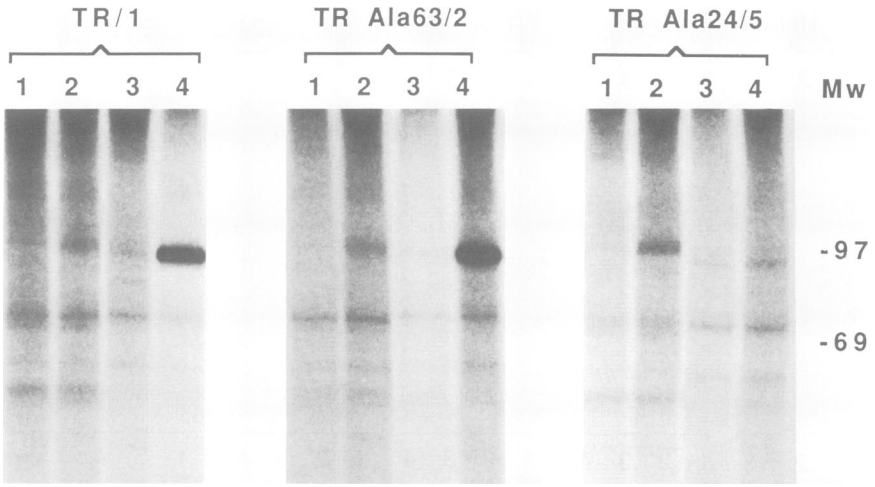

Fig. 3. Effect of phorbol ester on the phosphorylation state of mouse and human TRs in TR/1, TR Ala63/2 and TR Ala24/5 cells. Cells were labeled with [^{32}P]phosphate for 4 h and incubated for an additional 40 min without **(lanes 1,3)** or with **(lanes 2,4)** 100 nM PDBu. After solubilization, the mouse TR was first immunoprecipitated using the rat anti-mouse TR monoclonal antibody H.129.121 **(lanes 1–2)** followed by immunoprecipitation of the human TR with the PA-1 monoclonal antibody **(lanes 3–4)**. Reduced samples were analyzed on a 8% polyacrylamide gel. The autoradiogram of the dried gel was exposed for 24 h.

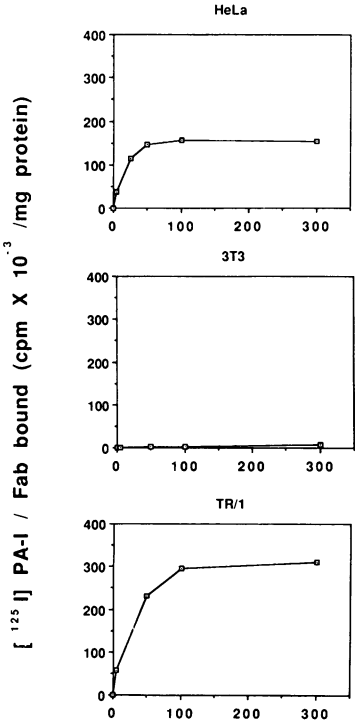

Fig. 4. Concentration-dependent binding of [^{125}I]PA-1/Fab fragment of HeLa, 3T3 and TR/1 cells. The iodinated Fab fragment (specific activity = 1.6×10^6 c.p.m./μg) was incubated at the indicated concentrations with the cells (duplicate samples) for 40 min at 4°C in DMEM medium containing 1 mg/ml BSA. After washing off excess unbound ligand, the cells were lysed and both radioactivity and protein content were determined.

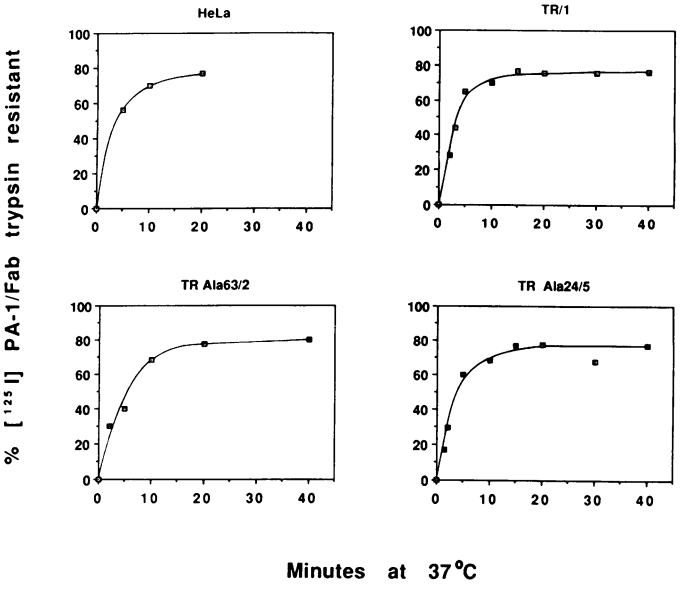

Fig. 5. Internalization of [^{125}I]PA-1/Fab fragment by HeLa, TR/1, TR Ala63/2 and TR Ala24/5 cells. The cells were incubated in the presence of 25 nM iodinated Fab fragment as described in Figure 4 and in Materials and methods. After washing off excess unbound ligand, the cells were incubated at 37°C for the indicated time and then treated with trypsin at 4°C. The internalized labeled Fab fragment is expressed as ratio between cell-associated counts and total radioactivity (cell-associated counts plus counts in the supernatant). The data are representative of three different experiments.

tor (see below and Figure 6). Since this would create a background problem for the analysis of the transformed lines (which contain both human and mouse TR), we decided to use iodinated Fab fragments of the anti-human TR monoclonal antibody PA-1. The Fab approach has already successfully been used to study the internalization and recycling of the Fc receptor (Mellman *et al.*, 1984a,b), the mannose-6-phosphate receptor (Gartung *et al.*, 1985) and of the asialoglycoprotein receptor (Schwartz *et al.*, 1986). Fab fragments were prepared by papain digestion from PA-1 antibody and were iodinated. First, we showed that these fragments specifically bind to cells expressing human TR. HeLa, TR/1 and 3T3 cells were incubated at 4°C with various concentrations of the iodinated Fab fragments for 1 h. As seen in Figure 4, [^{125}I]PA-1/Fab binds to HeLa and TR/1 cells reaching saturation at a concentration between 50 and 100 nM. In contrast, almost undetectable binding was observed with 3T3 cells. We

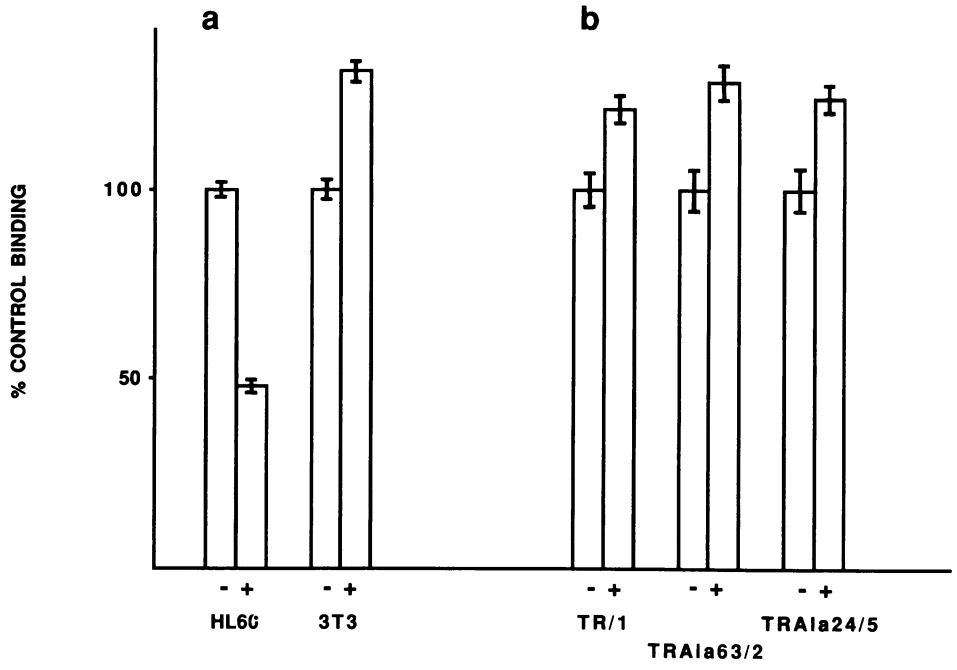

Fig. 6. Effect of phorbol ester dibutyrate on cell surface TR distribution. Cells were incubated in culture medium containing 1% fetal calf serum and 1 mM $CaNO_3$ at 37°C for 1 h in the absence (−) or in the presence (+) of 100 nM PDBu, as previously described (May *et al.*, 1985b). The histogram reports the results of ^{125}I-labeled human ferro-transferrin (50 nM 1 × 10^5 c.p.m./μg) binding to HL60 and 3T3 cells as control in **(a)** and TR/1, TR Ala63/2 and TR Ala24/5 cells in **(b)** at 4°C for 1 h. Values from stimulated cells are expressed as average per cent of those from unstimulated cells (control binding ± s.e.m.), arbitrarily set at 100%. Total specific binding of ^{125}I-labeled ferro-transferrin expressed as c.p.m. × 10^{-3}/mg protein had a value of 600 for HL60 cells, 50 for 3T3, 580 for TR/1, 480 for TR Ala63/2 and 400 for TR Ala24/5 cells.

conclude that our Fab fragment is specific for the human TR.

To follow the endocytosis and recycling of the human TR the iodinated Fab fragments were first bound to TR/1, TR Ala63/2, TR Ala24/5 and HeLa (control) cells for 40 min at 4°C. The cells (duplicate samples) were then incubated at 37°C for different periods of time to induce endocytosis. The amount of internalized and surface-bound Fab fragments was finally determined by treating the cells with trypsin and counting the radioactivity in the supernatant and cell-associated fraction after centrifugation of the cells. This treatment efficiently removes the surface-bound labeled ligand since in the absence of internalization (without incubation at 37°C) the trypsin digestion released 98% of the bound labeled Fab. The ratio between protease-protected (intracellular) and total (intracellular and released) [^{125}I]PA-1/Fab was plotted against the time of incubation at 37°C and the result is shown in Figure 5. As can be seen TR, TR Ala63 and TR Ala24 are all efficiently and rapidly internalized with kinetics very similar to that of TR in HeLa cells; 50% of the labeled Fab becomes trypsin resistant in 3.5 min and a steady-state pool of 75% internal TR−Fab complex is achieved within 15 min. This result is quite similar to that obtained by Watts (1985) in K562 cells. At 20, 30 and 40 min the same ratio is observed. This steady-state distribution is likely to be a consequence of recycling, in that at these time points the extent of internalized TR/Fab complexes is equal to that of complexes which are recycled back to the cell surface. If the TR/Fab complexes were routed to the lysosomes rather than being recycled to the cell surface, we would expect a progressive clearing of the TR−Fab complexes from the plasma membrane. Electrophoretic analysis of solubilized samples from cells incubated at 37°C for 5 or 40 min did not essentially indicate degradation of the internalized iodinated Fab fragments even at the later time point (data not shown).

From the kinetics of endocytosis and recycling of the mutant and control TRs we conclude that phosphorylation is not involved in the control of TR endocytosis and recycling.

Phorbol ester induces down-regulation of transferrin-binding sites in HL60 but not in 3T3 cells

We analyzed the effect of phorbol ester on the TR down-regulation in mouse 3T3, TR/1, TR Ala63/2, TR Ala24/5 and control HL60 cells. To determine the distribution of TR molecules on the cell surface ^{125}I-labeled human ferro-transferrin was bound at 4°C to cells previously incubated at 37°C for 1 h in the presence or absence of 100 nM PDBu. Unexpectedly no down-regulation of either the endogenous mouse TR (see Figure 6a) or the heterologous TR (Figure 6b) was observed in mouse 3T3 cells and in the transformed 3T3 cells stimulated by phorbol ester, although it occurred in HL60 cells as previously reported by others (May *et al.*, 1984, 1985a,b). There was an ~50% decrease of transferrin-binding sites in HL60 cells, whereas there was a 20−30% increase in the case of 3T3 cells and transformed clones. Analogous results were obtained using iodinated PA-1/Fab fragments as ligands for human TR (not shown). This lack of down-regulation was occurring in spite of an increase in the phosphorylation state as described before (Figure 3). These results suggest that phosphorylation does not influence the surface distribution of TR molecules at equilibrium in 3T3 cells and that phorbol ester-induced TR down-regulation is not a general feature of different cell types.

We can therefore conclude that the human TR behaves exactly as the endogenous mouse TR in 3T3 cells. The different effect of phorbol ester in HL60 and 3T3 cells must therefore be due to cell-specific factors rather than sequence differences in the TR molecules.

Discussion

In three different cell lines of human orign (HL60, A431 and K562), phorbol ester has been shown to increase the phosphorylation state of TR and to down-regulate the TR at the cell surface (Klausner *et al.*, 1984; May *et al.*, 1984, 1985a,b; Davis *et al.*, 1986). These results have led to the hypothesis that protein kinase C-mediated phosphorylation is a signal for induction and regulation of TR endocytosis and recycling. A similar effect of phorbol ester has also been reported for the epidermal growth factor receptor (Beguinot *et al.*, 1985; Fearn and King, 1985), suggesting that the effect on TR is not unique. However, the studies on endocytosis of other receptors have shown that the effect of phorbol ester cannot be generalized. For instance, phorbol esters seem to work quite differently on the asialoglycoprotein receptors compared with TR in the same cell type (Hep-G2). In HepG2 cells, the asialoglycoprotein receptor was also down-regulated but this was due much more to a phorbol ester-induced decrease in binding affinity rather than receptor redistribution (Fallon and Schwartz, 1986).

In this work, we have directly tested the role of TR phosphorylation as a signal for endocytosis and recycling. This was done by changing the phosphorylation site, Ser24, into a non-phosphorylatable residue (Ala24), and by analyzing the mutant phenotype expressed in 3T3 cells. The experiments performed on the TR Ala24 mutant, the wild-type and the control mutant TR (TR Ala63) in 3T3 cells, indicate that: (i) phorbol ester enhances the phosphorylation state of control TR but not of Ala24 mutant, and (ii) both wild-type and mutant TR receptors display a normal endocytosis and recycling behaviour. Thus, we conclude that protein kinase C phosphorylation of Ser24 does not constitute a signal or part of a signal required for endocytosis and recycling in these cells.

The finding that down-regulation of hyperphosphorylated TR does not occur in 3T3 cells, using the same phorbol ester treatment which responded positively in HL60 control cells, shows that phosphorylation is neither involved in regulating the efficiency of internalization nor recycling of TR in 3T3 cells (Davis *et al.*, 1987). Phorbol ester-induced down-regulation of TR in HL60 cells (and also the previously studied K562, A431 cells) seems to depend on the presence of cell type-specific factors which are sensitive to phorbol esters and control receptor internalization. However, it remains to be seen whether down-regulation in HL60 cells involves phosphorylated receptors. To answer this question, it would be important to test the behaviour of TR Ala24 in a non-human cell line where down-regulation of the endogenous receptor does occur. In this context it is interesting to note the recent work by Lin *et al.* (1986) on phorbol ester-induced EGF receptor down-regulation. The authors expressed the normal human EGF receptor and a mutant receptor lacking the phosphorylation site for protein kinase C (Thr654 → Ala) in heterologous cells (rat fibroblasts) and found that both molecules could be internalized (and degraded) following ligand binding. However, phorbol ester caused down-regulation of the wild-type receptor but not of the mutant one. Thus, in the case of the EGF receptor two different mechanisms seem to affect receptor internalization in these cells, one of them being dependent on receptor phosphorylation.

It is of course also possible that TR phosphorylation is totally unrelated to endocytosis and recycling in all cells, as it seems to be in 3T3 cells. This possibility does not rule out that this modification has another important function, unknown at present. An insight into this problem could be obtained by studying TR Ala24 in a mutant cell line which is devoid of functional endogenous TR.

Materials and methods

Materials

Restriction endonucleases, *Escherichia coli* DNA polymerase I (Klenow fragment), SP6 polymerase and *Xba*I linkers were obtained from Boehringer (Mannheim). The ultrapure deoxy- and dideoxyribonucleotides used for DNA sequencing were from Pharmacia, Freiburg. The antibiotic G418 was purchased from Gibco. The OKT9 antibody was from Ortho Diagnostics, New Jersey. The PA-1 antibody was provided by Gerd Moldenhauer [Deutsches Krebsforschungszentrum (DFKZ), Heidelberg, FRG] and the H.129.121 antibody was provided by Michel Pierres (Centre d'Immunologie de Marseille-Luminy, Marseille, France). Rabbit anti-mouse Ig and anti-rat Ig sera were obtained from Nordic Immunological Laboratories, Tilburg. Sephadex G50 and protein A – Sepharose CL-4B was obtained from Pharmacia. DE-52 was from Whatman, Maidstone. L-[^{35}S]Methionine, [^{33}P]phosphate and ^{125}I were purchased from Amersham (Braunschweig). The solid phase radioiodination reagent Iodo-Gen was from Pierce (Rockford).

DNA engineering and oligonucleotide site-directed mutagenesis

Nucleic acid methods for plasmid constructions were as described in Maniatis *et al.* (1982). *In vitro* mutagenesis was carried out using the two synthetic oligonucleotides (25 mers) shown in Figure 1a and b. As template DNA, a 0.9-kb *Hin*dIII – *Hin*dIII fragment encoding the first 292 terminal residues of TR was isolated from pGEM1 TR (Zerial *et al.*, 1986) and inserted into M13 mp19. The oligonucleotides are complementary to nucleotides 321 – 345 and 438 – 462 of the template cDNA (Schneider *et al.*, 1984) except for three mismatches which convert the Ser24 and Ser63 residues into Ala, respectively. After priming with the oligonucleotides 'all-the-way-around' extension by the Klenow large fragment of DNA polymerase I was carried out. Competent BMH 71-18 mut-L cells were transformed by the reaction products and the resulting infected colonies screened by differential hybridization to the 5′-$^{32}PO_4$-labeled oligonucleotides as described by Zoller and Smith (1983). Double-stranded phage DNA was prepared from putative mutants and used for transformation of BMH 71/18 cells. After rescreening by oligonucleotide hybridization, single-stranded phage DNA was prepared from positive colonies and the mutation verified by dideoxy sequencing (Sanger *et al.*, 1977).

Construction of pGEM1 TR Ala24 and pGEM1 TR Ala63

To ensure that no additional mutations were introduced, completely sequenced 324-bp *Xho*I – *Dde*I fragments containing the Ala24 and Ala63 mutations were cut from the double-stranded M13 mutant DNAs and inserted into pGEM1 TR to yield pGEM1 TR Ala24 and pGEM1 TR Ala63. The *Xho*I – *Dde*I fragments encode the complete (mutated) cytoplasmic tail, the transmembrane region and a few amino acids of the TR ectodomain. The two plasmids were constructed by performing a four-fragment ligation. Besides the two mutated *Xho*I – *Dde*I fragments mentioned above, the following three fragments isolated from pGEM1 TR were common to both plasmids: fragment *Dde*I (position 550 in the TR map, Schneider *et al.*, 1984) to *Hin*dIII (position 1079 in the TR map), fragment *Hin*dIII – *Pvu*I (in the β-lactamase gene of pGEM1) and fragment *Pvu*I – *Xho*I (in the polylinker of pGEM1). MC1061 transformants were first analyzed by restriction mapping and then checked for maintenance of reading frame by *in vitro* transcription – translation as previously described (Zerial *et al.*, 1986, 1987).

Construction of pSV TR, pSV TR Ala24 and pSV TR Ala63

For the purpose of expression in eucaryotic cells, wild-type and mutant TR cDNAs were excised as *Eco*RI fragments from pGEM1 vectors and, after the addition of *Xba*I linkers, inserted into the *Xba*I site of the SV40 early-replacement vector pSVd2-3 tss$^-$ (D.Huylebroeck, unpublished).

Isolation of mouse 3T3 transfected cell lines

Mouse 3T3 cells were co-transfected with either plasmid (10 μg) and with pSV Neo (0.5 μg) by using the calcium phosphate precipitation method (Graham and van der Eb, 1973). After 48 h the cells were grown in selective medium (DMEM containing 10% FCS, 10 mM Hepes pH 7.4, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) containing 1 mg/ml G418. After 15 days clear colonies were visible. They were propagated and screened by indirect immunofluorescence staining using the anti-human TR monoclonal antibody OKT9 followed by rhodamine-conjugated goat anti-mouse immunoglobin. Three transformant cell lines called TR/1, TR Ala63/2 and TR Ala24/5 were chosen for the studies and were propagated in culture medium containing 1 mg/ml G418.

Sequential immunoprecipitation of human and mouse TR

Dishes (10 cm) with sub-confluent TR/1, TR Ala63/2, TR Ala24/5 cells and HeLa and 3T3 as control cells were labeled with 150 μCi of [^{35}S]methionine (1000 Ci/mmol) per ml of Met-free DMEM for 6 h at 37°C. The cells were then lysed

in 1 ml of 1% Nonidet P-40, 50 mM Tris−HCl pH 7.5, 150 mM NaCl, 2 mM EDTA and 20 μg/ml PMSF. For immunoprecipitation 100-μl aliquots were used. After pre-clearing with a rabbit pre-immune serum, they were incubated with 4 μg of the mouse anti-human TR monoclonal antibody PA-1 [Moldenhauer, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, FRG] for 2 h in the presence of rabbit anti-mouse Ig serum and protein A−Sepharose CL-4B. This step was repeated once more to ensure that the lysate was devoid of human TR. Next, the mouse TR was immunoprecipitated by 4 μg of the rat anti-mouse TR monoclonal antibody H.129.121 (Van Agthoven *et al.*, 1984) in the presence of rabbit anti-rat Ig serum and protein A−Sepharose CL-4B. This step was repeated once more. The material bound to the Sepharose beads was then solubilized and divided into two aliquots: one of these was heated to 95°C for 5 min after addition of DTT and alkylated with iodacetamide (reducing conditions); the other was just heated to 70°C for 3 min (non-reducing conditions). Samples were analyzed by 8 and 6% SDS−PAGE and processed for fluorography. The experiment was also performed with the opposite order of immunoprecipitation and yielded the same results.

Treatment of cells with phorbol ester and [^{125}I]transferrin and [^{125}I]PA-1/Fab binding

Cells in 2.5-cm dishes were treated with 100 nM PDBu for 60 min as described in May *et al.* (1985). They were then incubated with either 50 nM ^{125}I-labeled human ferro-transferrin or 25 nM [^{125}I]PA-1/Fab fragment (see below) in 50 mM Tris−HCl pH 7.5, 150 mM NaCl, 1 mg/ml Ig-free BSA for 60 min at 4°C. Cells were washed twice with binding buffer for 5 min and solubilized in 400 μl 0.6% TX-100, 50 mM Tris−HCl pH 7.5, 150 mM NaCl, 2 mM EDTA. Radioactivity was counted in a γ counter and protein content determined for each sample using the Bio Rad (München) assay.

Sequential immunoprecipitation of ^{32}P-labeled mouse and human TR

TR/1, TR Ala63/2 and TR Ala24/5 cells were incubated in phosphate-free DMEM in the presence of 1 mCi/ml [^{32}P]orthophosphoric acid for 3 h at 37°C as described in May *et al.* (1985). The cells were further incubated at 37°C for 60 min in the presence or absence of 100 nM PDBu. The monolayers were washed once and lysed with 0.5 ml 1.5% TX-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 100 μM Na_3VO_5, 2 mM PMSF, 10 μg/ml leupeptin and 20 mM Hepes pH 7.5 (Davis *et al.*, 1986). Half of the lysate was used for immunoprecipitation. First, the mouse TR was immunoprecipitated by H.129.121 antibody and then the human TR was isolated using PA-1 antibody. The reduced samples were electrophoresed on an 8% polyacrylamide gel in the presence of 0.1% SDS.

[^{125}I]PA-1/Fab binding and human TR endocytosis assay

Fab fragments were prepared from PA-1 antibody by papain digestion (1 μg papain/100 μg PA-1) for 1 h. Separation of the Fab from Fc fragments and undigested antibodies was performed by DE-52 chromatography (Whatman). Iodination of the fragment was carried out using the Iodogen system. For binding studies, the cells (duplicate samples) grown on 2.5-cm dishes were washed twice with cold PBS and incubated with 300 μl DMEM containing 1 mM Hepes pH 7.4, 1 mg/ml Ig-free BSA and various concentrations of iodinated PA-1/Fab fragments at 4°C for 60 min. Monolayers were washed three times for 5 min with cold PBS before being lysed with 0.5 ml of 0.6% TX-100, 50 mM Tris−HCl pH 7.5, 150 mM NaCl and 2 mM EDTA. Both radioactivity and protein content were determined for each sample. When endocytosis had to be induced, after incubation with 25 nM [^{125}I]PA-1/Fab at 4°C for 60 min and 3 × 5-min washes with cold PBS, the cells (duplicate samples) were incubated in DMEM containing 1 mM Hepes pH 7.4 and 1 mg/ml Ig-free BSA at 37°C for different periods of time. Endocytosis was arrested by quickly removing the medium and adding cold PBS (two washes). The cells were then treated with 0.5 mg/ml trypsin in 0.15 M NaCl, 1 mM EDTA, 20 mM Hepes pH 7.4 at 4°C for 60 min as previously described (Watts, 1985). Following proteolysis, the cells detached and were then pelleted by centrifugation at 3000 r.p.m. for 3 min in a Biofuge A centrifuge (Heraeus). Radioactivity in cell pellets and supernatant was determined using a γ counter. Without any incubation at 37°C after iodinated Fab fragment binding, ~98% of surface-bound ligand could be released by the protease treatment.

Acknowledgements

We are indebted to Gerd Modlenhauer (Heidelberg) and Michel Pierres (Marseille) for their generous gifts of anti-human TR (PA-1) and anti-mouse TR (H.129.121) monoclonal antibodies, respectively; and to Danny Huylebroeck for the pSVd 2-3 tss$^-$ expression vector. We wish to thank especially Stephen Fuller and Jean Gruenberg for many helpful discussions and valuable suggestions and David Meyer, Raffaele Matteoni, Jean Davoust and Elizabeth Sztul for their critical reading of the manuscript. We gratefully acknowledge Tuula Manninen whose expert technical assistance has been determinant for the experiments, Anne Walter for typing the manuscript and Perla Zerial for the drawings. Marino Zerial and Maarit Suomalainen were recipients of EMBO long-term and EMBL short-term fellowships, respectively.

References

Beguinot,L., Hanover,J.A., Ito,S., Richert,N.D., Willingham,M.C. and Pastan,I. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2774−2778.
Bleil,J.D. and Bretscher,M.S. (1982) *EMBO J.*, **1**, 351−355.
Booth,A.G. and Wilson,M.J. (1981) *Biochem. J.*, **196**, 355−362.
Castagna,M., Takay,Y., Kaibucki,K., Sano,K., Kikkawa,U. and Nishizuka,Y. (1982) *J. Biol. Chem.*, **257**, 7847−7851.
Ciechanover,A., Schwartz,A.L., Dautry-Varsat,A. and Lodish,H.F. (1983) *J. Biol. Chem.*, **258**, 9681−9689.
Colbere-Garapin,F., Horodniceanu,F., Kourilski,P. and Garapin,A. (1981) *J. Mol. Biol.*, **150**, 1−14.
Dautry-Varsat,A., Ciechanover,A. and Lodish,H.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2258−2262.
Davis,R.J., Johnson,G.L., Kelleher,D.J., Anderson,J.K., Mole,J.E. and Czech, M.P. (1986) *J. Biol. Chem.*, **261**, 9034−9041.
Davis,C.G., van Driel,I.R., Russell,D.W., Brown,M.S. and Goldstein,J.L. (1987) *J. Biol. Chem.*, **262**, 4075−4082.
Gartung,C., Braulke,T., Hasilik,A. and von Figura,K. (1985) *EMBO J.*, **4**, 1725−1730.
Fallon,R.J. and Schwartz,A.L. (1986) *J. Biol. Chem.*, **261**, 15081−15089.
Fearn,J.C. and King,A.C. (1985) *Cell*, **40**, 991−1000.
Graham,G.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 457−476.
Harding,C., Henser,J. and Stahl,P. (1983) *J. Cell Biol.*, **97**, 329−339.
Hopkins,C.R. (1983) *Cell*, **35**, 321−330.
Hopkins,C.R. and Trowbridge,I.S. (1983) *J. Cell Biol.*, **97**, 508−521.
Jing,S. and Trowbridge,I.S. (1987) *EMBO J.*, **6**, 327−331.
Klausner,R.D., Ashwell,G., van Renswoude,J., Harford,J.B. and Bridges,K.R. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2263−2266.
Klausner,R.D., Harford,J. and van Renswoude,J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3005−3009.
Lin,C.R., Chen,W.S., Lazar,C.S., Carpenter,C.D., Gill,G.N., Evans,R.M. and Rosenfeld,M.G. (1986) *Cell*, **44**, 838−848.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
May,W.S. and Cuatrecasas,P. (1985) *J. Membr. Biol.*, **88**, 205−215.
May,W.S., Jacobs,S. and Cuatrecasas,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2016−2020.
May,W.S., Sahyoun,N., Jacobs,S., Wolf,M. and Cuatrecasas,P. (1985a) *J. Biol. Chem.*, **260**, 9419−9426.
May,W.S., Sahyoun,N., Wolf,M. and Cuatrecasas,P. (1985b) *Nature*, **317**, 549−551.
McClelland,A., Kuhn,L. and Ruddle,F.H. (1984) *Cell*, **39**, 267−274.
Mellman,I., Plutner,H. and Ukkonen,P. (1984a) *J. Cell Biol.*, **98**, 1163−1169.
Mellman,I. and Plutner,H. (1984b) *J. Cell Biol.*, **98**, 1170−1177.
Newman,R., Domingo,D., Trotter,J. and Trowbridge,I. (1983) *Nature*, **304**, 643−645.
Octave,J.N., Schneider,Y.J., Crichton,R.R. and Trouet,A. (1981) *Eur. J. Biochem.*, **115**, 611−618.
Pearse,B.M.F. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 451−455.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.
Schneider,C., Owen,M.J., Banville,D. and Williams,J.G. (1984) *Nature*, **311**, 675−678.
Schwartz,A.L., Ciechanover,A., Merritt,S. and Turkewitz,A. (1986) *J. Biol. Chem.*, **261**, 15225−15232.
Sibley,D.R., Benovic,J.L., Caron,M.G. and Lefkowitz,R.J. (1987) *Cell*, **48**, 913−922.
Van Agthoven,A., Goridis,C., Naquet,P., Pierres,A. and Pierres,M. (1984) *Eur. J. Biochem.*, **140**, 433−440.
Watts,C. (1985) *J. Cell Biol.*, **100**, 633−637.
Willingham,M.C., Hanover,J.A., Dickson,R.B. and Pastan,I. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 175−179.
Zerial,M., Melançon,P., Schneider,C. and Garoff,H. (1986) *EMBO J.*, **5**, 1543− 1550.
Zerial,M., Huylebroeck,D. and Garoff,H. (1987) *Cell*, **48**, 147−155.
Zoller,M.J. and Smith,M. (1983) *Methods Enzymol.*, **100**, 468−501.

Received on May 12, 1987

Note added in proof

After this paper was submitted, Rothemberg,S., Iacopetta,B.J. and Kühn,L. (1987) *Cell*, **49**, 423−431, have reported a similar study on the human TR. Their results are in agreement with those reported in the present paper.