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Direct Linkage of Thrombin to Its Cell Surface Receptors in Different Cell Types

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When ^{125}I-thrombin was incubated with foreskin fibroblasts, cervical carcinoma cells or fibrosarcoma cells of human origin, or with secondary chick embryo cells or Chinese hamster lung cells, it became directly linked to its cell surface receptors. The thrombin-receptor complex (TH-R) was derived exclusively from a pool of ^{125}I-thrombin that had become specifically bound to the cell surface. The linkage was probably covalent, since the complex was resistant to boiling in sodium dodecyl sulfate and 2-mercaptoethanol. Raising the pH to 12 disrupted TH-R, but did not affect a similar complex between epidermal growth factor and its receptor, suggesting that the linkage of these mitogens to their receptors was different. Mild trypsin treatment removed the ability of cells to form TH-R; however, after a 24-h incubation in serum-free medium, trypsin-treated cells recovered the capacity to form TH-R, suggesting that TH-R resulted from interaction of ^{125}I-thrombin with a cellular rather than a serum component. The mitogenic response of cells to thrombin was inversely related to the fraction of specifically bound ^{125}I-thrombin represented by TH-R. The role of TH-R in mitogenesis may be clarified in future studies by obtaining clones of Chinese hamster lung cells that vary in their capacities to form TH-R and to respond to the mitogenic action of thrombin.

Key words: **thrombin receptors, epidermal growth factor receptors, cell proliferation, normal and transformed cells**

Recently it has been shown that when ^{125}I-labeled epidermal growth factor (^{125}I-EGF) was incubated with human [1] or mouse [2] fibroblasts, about 1–10% of the EGF molecules that were specifically bound to cell surface receptors became linked to them. The EGF-receptor complex (EGF-R) was resistant to boiling in 3% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol, suggesting that the linkage was probably covalent.

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EGF-R was formed at the cell surface and with time was internalized and cleaved to discrete-molecular-weight forms. The complex and subsequent forms were very similar to ones seen by Das and Fox [3] and Das et al [4], who used a photoactivable derivative of ^{125}I-EGF to crosslink EGF to its receptor and follow the fate of this complex during continued incubation of cells.

It was also reported that incubation of another polypeptide mitogen, ^{125}I-thrombin (^{125}I-TH), with human fibroblasts led to formation of a complex between TH and its cell surface receptor [1]. Like EGF-R, the TH-receptor complex (TH-R) was resistant to boiling in 3% SDS and 1% 2-mercaptoethanol. However, an intriguing difference between EGF-R and TH-R formation in the human cells was that up to 60% of specifically bound TH became linked to its receptor, whereas the corresponding amount for EGF was only 6–9%.

These results prompted the present investigations on TH-R in several kinds of cells. We examined the amounts of TH-R formed and compared this with the ability of the cells to respond to the mitogenic action of TH. We also determined the molecular weights of TH-R in the different cells and showed that TH-R was not derived from a serum component. In addition, we examined the nature of the linkages between the mitogen-receptor complexes, and we report that the linkage for TH-R is different from the EGF-R linkage.

MATERIALS AND METHODS

Purified human thrombin was provided by Dr. John W. Fenton II [5]. EGF was purified from male mouse submaxillary glands by the procedure of Savage and Cohen [6]. MCDB medium 202 was prepared according to Ham and McKeehan [7]. Dulbecco-Vogt modified Eagle's medium (DV) and tryptose phosphate broth were obtained from Flow Laboratories, Inc. Calf serum was purchased from Irvine Scientific. Other medium products, chicken serum, and trypsin solution were purchased from Gibco. Tissue culture dishes were obtained from Falcon Plastics. Na^{125}I was obtained from Amersham, lactoperoxidase from Calbiochem, and bisbenzimide #33258 (Hoechst dye) from American Hoechst.

Normal human fibroblasts (HF cells) prepared from neonatal foreskin explants were maintained as previously described [1]. Cells at passages 7–13 were used for experiments. D98/AH-2 cells (a variant of the Hela human cervical carcinoma line) and HT1080 cells (a human fibrosarcoma cell line) were provided by Dr. Eric J. Stanbridge. Chinese hamster lung (CHL) fibroblasts from the V79 strain [8] were provided by Dr. John J. Wasmuth. Cultures of chick embryo (CE) cells were prepared by the method of Rein and Rubin [9]. Primary cultures were grown in DV medium containing 2% chicken serum and 2% tryptose phosphate broth. Confluent primary cultures were subcultured at 5.7×10^4 cells/cm^2 in fresh medium. After 4 h, these secondary cultures were rinsed with serum-free DV medium (DV-0) and then incubated for 48 h in DV-0 before use in ^{125}I-TH binding experiments. All other cells were grown in DV medium containing 5% calf serum (DV-5). All cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. Stock cultures were seeded at 5.8×10^3 cells/cm^2 (HF cells), 1.5×10^4 cells/cm^2 (CHL and HT1080 cells) and 3.3×10^4 cells/cm^2 (D98 cells). To determine their mitogenic response to TH, CHL and HT1080 cells were plated at 2.3×10^4 cells/cm^2 in DV-5. After 2 days, the medium was replaced with DV-0 or DV medium containing 0.1% calf serum (DV-0.1) for CHL and HT1080 cultures, respectively. Two days later TH was added to final concentrations of 0.3 μg/ml or 3.0 μg/ml, and cell number was determined 48 h

later. The effect of TH on cell division was determined by comparing cell number in TH-treated cultures with parallel untreated cultures.

D98, HF, HT1080, and CHL cells were judged to be free of mycoplasma contamination by a modification of the method of Chen [10]. Briefly, subconfluent cultures growing on glass coverslips were fixed in phosphate-buffered saline containing 3% (w/v) formaldehyde, stained in 2 μg/ml Hoechst dye and then analyzed by fluorescence microscopy for mycoplasma.

TH was iodinated by the lactoperoxidase method of Martin et al [11]. EGF was iodinated by the procedure of Carpenter and Cohen [12]. ^{125}I-EGF and ^{125}I-TH total and nonspecific binding to cells was measured as previously described [1] unless noted otherwise. After unbound ^{125}I-EGF or ^{125}I-TH was rinsed from cells with ice-cold phosphate-buffered saline, the cells were solubilized in 0.2 ml solubilization buffer [3% (w/v) SDS, 0.6% (w/v) N-ethylmaleimide, 100 mM Tris-Cl (pH 6.8), 1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 2 mM phenylmethylsulfonyl fluoride, and 0.005% (w/v) bromophenol blue] and placed in a boiling water bath for 5 min. Where protein concentration was determined, the bromophenol blue was omitted. Samples were electrophoresed in 0.1% SDS, 7.5–15% linear gradient polyacrylamide slab or tube gels based on the method of Laemmli [13]. Tube gels were cut into 2-mm slices and the ^{125}I radioactivity in each slice was measured in a gamma counter. The slab gels were fixed, stained, and then dried on Whatman No. 1 chromatography paper. The dried gels were exposed to Kodak X-Omat R film in the presence of Dupont Cronex Lightning Plus intensifying screens. Protein concentrations of nonreduced samples were determined by the method of Lowry et al [14] and those of reduced samples by a modification of the Lowry assay [15].

RESULTS

Formation of TH-R in Different Cell Types

A first step in evaluating the ability of different cells to form TH-R was to determine if they had specific receptors for TH. When HF, HT1080, D98, 2°CE, and CHL cells were incubated with ^{125}I-TH, the fraction of binding that was specific ranged from 25 to 70% and varied with cell type and ^{125}I-TH concentration (Table I). To measure the ability of cells to form TH-R, we incubated them with ^{125}I-TH and solubilized and boiled the extracts in 3% SDS and 1% 2-mercaptoethanol before electrophoresis on SDS polyacrylamide gels. Autoradiographs of slab gels demonstrated that CHL (Fig. 1, lane 1), D98 (Fig. 1, lane 3), HT1080 (Fig. 1, lane 5), and 2°CE (Fig. 2, lane 7) cells formed complexes that were roughly similar in molecular weight to TH-R previously observed [1] in HF cells (Fig. 2, lane 9). We quantitated the contribution of TH-R to specific binding of ^{125}I-TH by electrophoresis of samples on SDS polyacrylamide tube gels and analysis of the distribution of ^{125}I radioactivity in 2-mm slices of the gels (Fig. 3 and Table I). As previously reported for HF cells, most of the nonspecifically bound radioactivity migrated with ^{125}I-TH or smaller-molecular-weight species (presumably due to degradation of ^{125}I-TH), while none of it migrated with TH-R. This suggested that TH-R was formed only from ^{125}I-TH that was specifically bound to cellular receptors. Although all cell types specifically bound ^{125}I-TH and formed TH-R, the percentage of specific binding due to TH-R varied among cell types. With HF and HT1080 cells TH-R formation was generally 4–10 times higher than with 2°CE, CHL, and D98 cells. These differences prompted us to examine the ability of TH to stimulate cell division in these cells.

TH-R and Stimulation of Cell Division

To determine if there was a relationship between TH-R formation and the mitogenic response of cells to TH, we added TH to nondividing cultures of CHL cells in DV-0 and HT1080 cells in DV-0.1, as described in Materials and Methods. Unfortunately, the mitogenic effect of TH could not be determined for D98 cells, since they continued to divide in DV-0.1 and, like HT1080 cells, they began to detach from the plates when placed in DV-0 for 24 h. Exposure of CHL cultures to TH at 0.3 μg/ml or 3.0 μg/ml increased cell number by 39% and 70%, respectively, after two days (Table II). This response was similar in magnitude to that reported by Carney et al [16] for 2°CE and 2° mouse embryo cells. In contrast, HT1080 cells, when treated with 3.0 μg/ml TH, increased in cell number by only 20% after 2 days. This response, albeit faster than that seen with HF cells, was no greater after 4 days of incubation with TH. Thus, HF and HT1080 cells, in which a large fraction of specifically bound ^{125}I-TH became linked to receptors, were mitogenically less responsive to TH than 2°CE and CHL cells, in which less linkage occurred.

Molecular Weights of TH-R in Different Cells

Analysis of autoradiographs such as those in Figures 1 and 2 revealed that the molecular weight of TH-R was not the same in all cell types. TH-R in both normal and transformed human cells had a molecular weight of about 68K as determined by comparison of their migration on SDS polyacrylamide gels with that of molecular weight standards. The complex was smallest in 2°CE cells, with an estimated molecular weight of 65K. In CHL fibroblasts the molecular weight of 76K for TH-R was closest to that previously reported [17] for another rodent, mouse, where TH-R was 80K. The cause of these differences in molecular weights of TH-R is unknown.

TABLE I. Specific Binding of ^{125}I-TH and Formation of TH-R

Cell type	^{125}I-TH concentration (ng/ml)	% Specific binding	% TH-R
A CHL	25	33	19
D98	100	29	11
HT1080	100	64	30
HF	25	67	36
HF	100	63	43
B CHL	200	50	13
D98	200	43	4.3
HT1080	200	25	35
2°CE	200	35	10
HF	200	66	43

Cells were incubated for 60 min at 37°C with the indicated concentrations of ^{125}I-TH. Specific binding was determined by subtracting the cpm bound in the presence of 8 μg/ml nonlabeled TH from the cpm bound in the absence of excess nonlabeled TH and then dividing this value by the cpm bound in the absence of excess nonlabeled TH and multiplying by 100%. Percentage TH-R was quantitated by electrophoresis of samples on SDS polyacrylamide tube gels, cutting the gels into 2-mm slices, and determining the ratio of specifically bound cpm in slices containing TH-R to the total number of specifically bound cpm in the entire gel (see Fig. 3). A, Cells prepared as in Figure 1; B, cells prepared as in Figure 2.

Complexes Between TH and Serum Components

In the course of our experiments we noted that often two bands, of molecular weights 77K and 90K, were also formed. Since these bands were present when cells growing in 5% serum were used (eg, Fig. 2, lane 3), yet absent when the cells had been in DV-0 or DV-0.1 for one day prior to incubation with ^{125}I-TH (Fig. 1, lane 3), we determined if

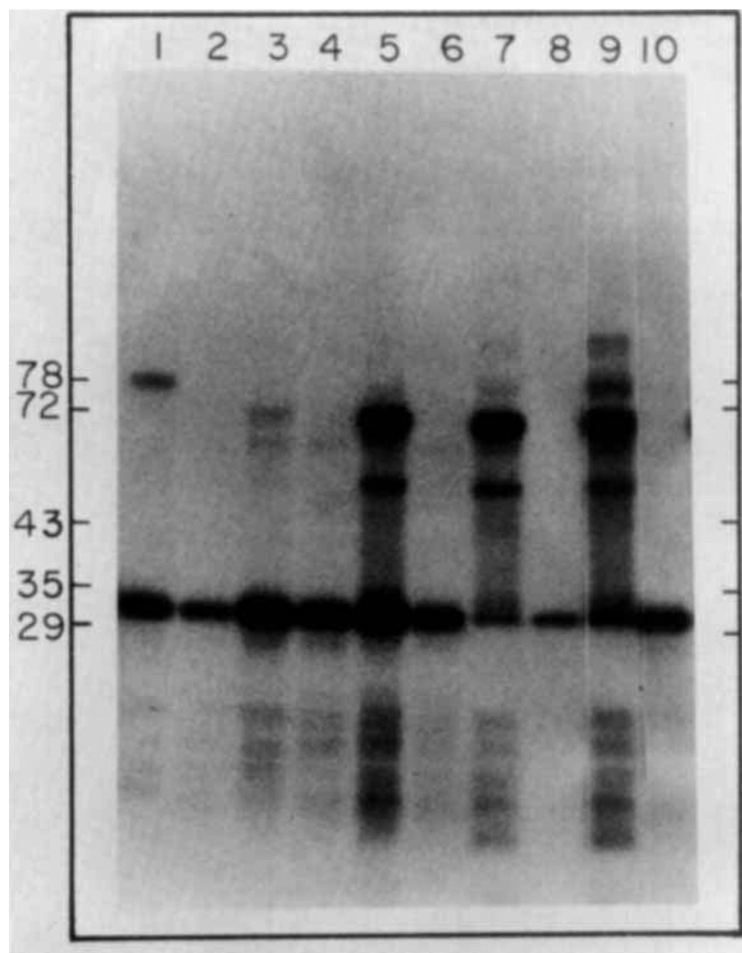

Fig. 1. Cells were seeded at 2 × 10^5 cells per 35-mm dish in DV-5. After reaching confluence, all cells except HF cells were placed in DV-0 for at least 2 days. Cells were incubated with ^{125}I-TH for 60 min at 37°C at the concentrations noted below. Equal volumes of each sample were used for electrophoresis. Lane 1, CHL cells incubated with 25 ng/ml ^{125}I-TH; lane 2, CHL cells incubated with 25 ng/ml ^{125}I-TH plus 8 μg/ml nonlabeled TH; lane 3, D98 cells incubated with 100 ng/ml ^{125}I-TH; lane 4, D98 cells incubated with 100 ng/ml ^{125}I-TH plus 8 μg/ml nonlabeled TH; lane 5, HT1080 cells incubated with 100 ng/ml ^{125}I-TH; lane 6, HT1080 cells incubated with 100 ng/ml ^{125}I-TH plus 8 μg/ml nonlabeled TH; lane 7, HF cells incubated with 25 ng/ml ^{125}I-TH; lane 8, HF cells incubated with 25 ng/ml ^{125}I-TH plus 8 μg/ml nonlabeled TH; lane 9, HF cells incubated with 100 ng/ml ^{125}I-TH; and lane 10, HF cells incubated with 100 ng/ml ^{125}I-TH plus 8 μg/ml nonlabeled TH.

^{125}I-TH might form complexes with serum components that were resistant to boiling in 3% SDS and 1% 2-mercaptoethanol. Figure 4 shows that 77K and 90K dalton complexes were indeed formed after incubation of 100 ng/ml ^{125}I-TH with 1% calf serum. Moreover, addition of 8 μg/ml nonlabeled TH diminished the amounts of the complexes, indicating that specific binding was involved. Actual identification of the factor(s) in serum that formed these complexes with ^{125}I-TH was not pursued; however, the molecular weights of the complexes and the fact that they were resistant to boiling in SDS and 2-mercaptoethanol indicated that they might result from linkage of TH with antithrombin III, a well-characterized serum component [18, 19].

Although TH-R formation was observed after maintenance of cells for 2 days in DV-0, the above results indicated that it was important to determine whether ^{125}I-TH was

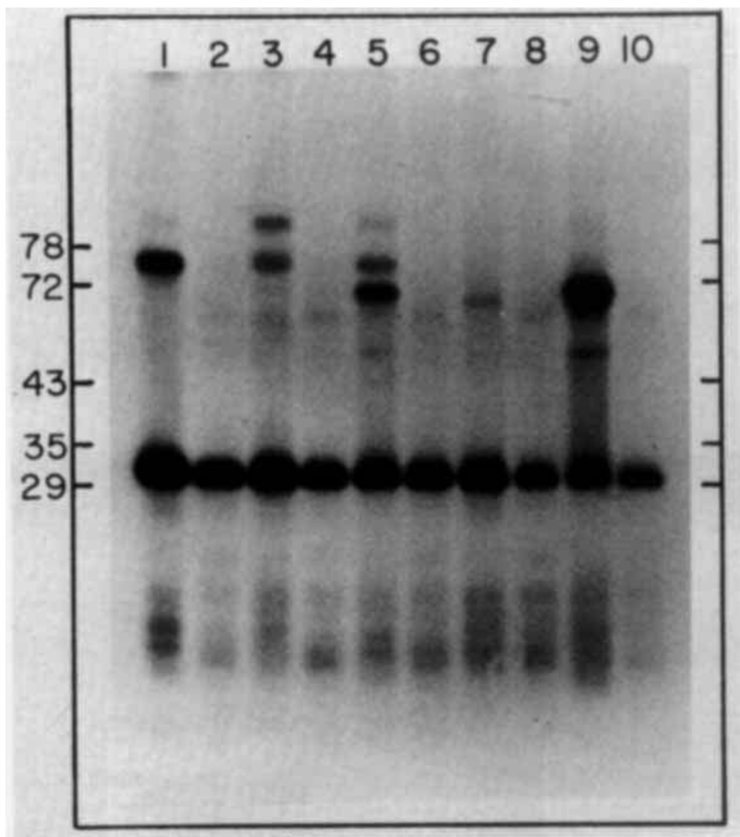

Fig. 2. 2°CE cells were prepared as described in Materials and Methods. All other cells prepared as in Figure 1 except that there was no medium change. Cells were incubated with 200 ng/ml ^{125}I-TH for 60 min at 37°C. Equal volumes of each sample were used for electrophoresis. Lane 1, CHL cells; lane 3, D98 cells; lane 5, HT1080 cells; lane 7, 2°CE cells; and lane 9, HF cells. Lanes 2, 4, 6, 8, and 10 represent binding of ^{125}I-TH under nonspecific binding conditions for CHL, D98, HT1080, 2°CE, and HF cells, respectively.

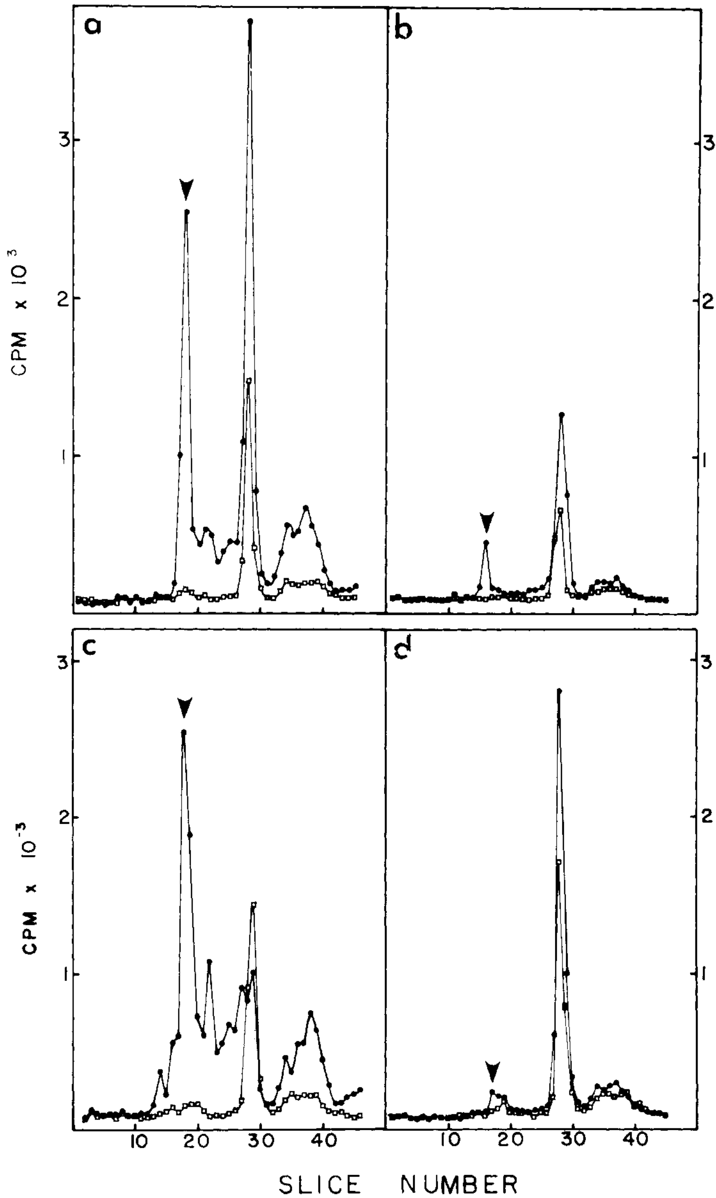

Fig. 3. Distribution of ^{125}I radioactivity in SDS polyacrylamide gels of samples from cells incubated for 60 min at 37°C with ^{125}I-TH at the concentrations noted below. Cells were prepared as in Figure 1. Arrowheads indicate the position of TH-R. Unlinked ^{125}I-TH migrated in gel slices 27–29. Panel a, HT1080 cells incubated with 100 ng/ml ^{125}I-TH; panel b, CHL cells incubated with 25 ng/ml ^{125}I-TH; panel c, HF cells incubated with 25 ng/ml ^{125}I-TH; panel d, D98 cells incubated with 100 ng/ml ^{125}I-TH. •, total binding of ^{125}I-TH; ▫, nonspecific binding of ^{125}I-TH.

TABLE II. Stimulation of Cell Division in CHL and HT1080 Cells by Thrombin

Cell type	TH concentration (μg/ml)	Cell number ($\times 10^{-6}$)	% Increase
CHL	–	1.33	–
CHL	0.3	1.85	39
CHL	3.0	2.26	70
HT1080	–	1.01	–
HT1080	0.3	1.04	3
HT1080	3.0	1.21	20

Cell numbers are averages of duplicate plates.

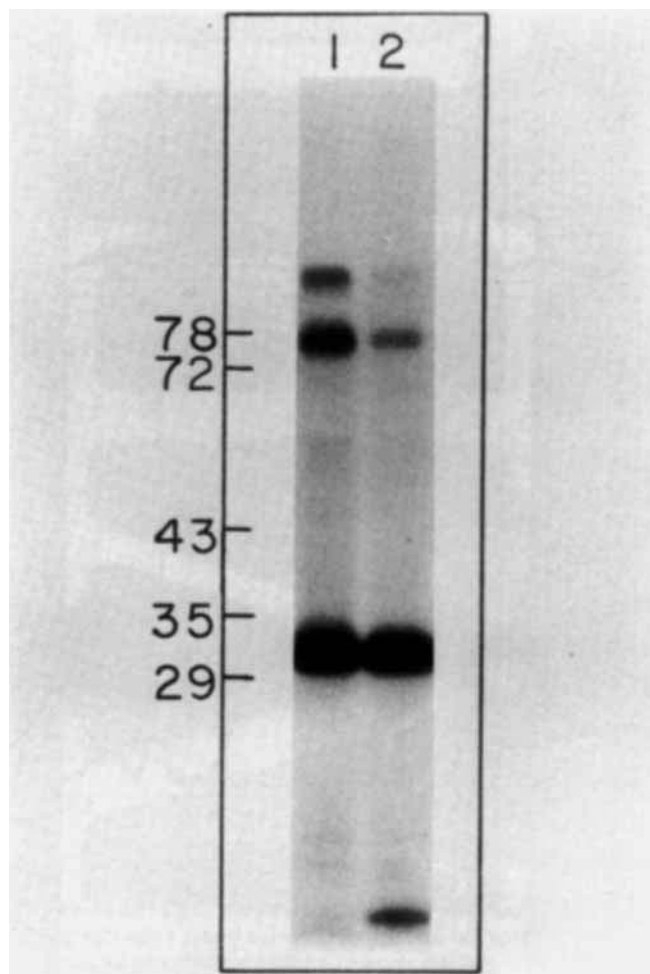

Fig. 4. Binding of ^{125}I-TH to calf serum. Lane 1: Binding buffer containing 1% calf serum and 100 ng/ml ^{125}I-TH was incubated for 60 min at 37°C. Five volumes of solubilization buffer were added and the sample was heated, electrophoresed, and exposed to film as described in Materials and Methods. Lane 2: Incubation in the presence of 8 μg/ml nonlabeled TH.

forming a complex with a cellular receptor or with a serum component that adsorbed to cells. Since TH-R could be removed by trypsin treatment of cells shortly after binding of ^{125}I-TH [1], it seemed reasonable that pretreatment with trypsin would remove (or change) the receptor for TH and in so doing prevent the formation of TH-R. If cells could then regenerate their capacity to form TH-R in serum-free medium, this would indicate that a cellular component was necessary for TH-R formation. As shown in Figure 5, lane

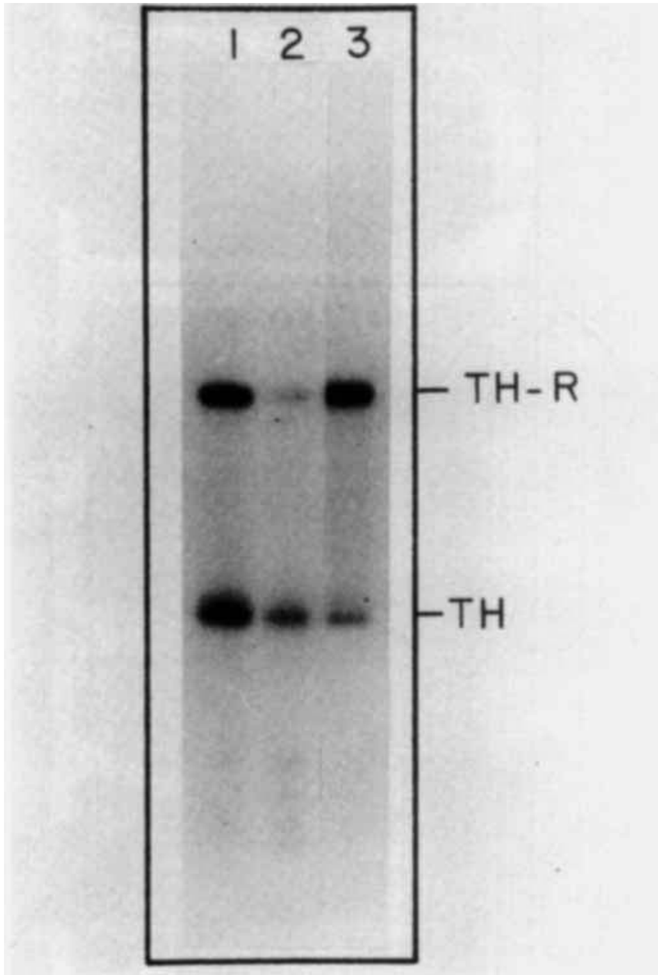

Fig. 5. TH-R formation within 1 h (lane 2) or 25 h (lane 1) after trypsin treatment or in untreated control cells (lane 3). HF cells were seeded at 2 × 10^5 cells per 35-mm dish in DV-5. Confluent cultures were rinsed once with 2.0 ml DV-0 and then incubated in DV-0 for at least one day. Cells in the recovery group were incubated at 37°C in DV-0 containing 500 μg/ml trypsin. After 5 min, cells were rinsed twice with 1.0 ml DV-0 containing 10 μg/ml soybean trypsin inhibitor (SBTI) and then incubated in a serum-free mixture of DV medium:MCDB 202 medium (2:1). The following day a second group of cells (lane 2) was trypsinized as noted above for 5 min. All trypsin-treated cells were then washed three times in DV-0 containing 10 μg/ml SBTI and incubated for 10 min at 23°C. Next, all three experimental groups of cells were washed three times in DV-0 prior to incubation for 30 min at 37°C with DV-0 containing 0.5% bovine serum albumin and 100 ng/ml ^{125}I-TH. Solubilization and electrophoresis of samples was performed as described in Materials and Methods. An equal amount of protein was loaded in each lane.

2, a 5-min treatment of HF cells with 500 μg/ml trypsin at 37°C drastically reduced the formation of TH-R compared to untreated cells (lane 3) when assayed within 1 h of trypsin treatment. Moreover, when trypsin-treated cells were placed in serum-free medium for 24 h, their ability to form TH-R returned (Fig. 5, lane 1). The simplest interpretation of these results is that TH-R was formed by the interaction of ^{125}I-TH with a cellular rather than a serum component.

Nature of the Mitogen-Receptor Linkage

Since incubation of mouse [2] and human [1] cells with ^{125}I-EGF also resulted in the formation of a mitogen-receptor complex that was resistant to boiling in SDS and 2-mercaptoethanol, it was important to determine whether EGF-R and TH-R were formed by the same or different mechanisms. Although we have not studied the types of chemical bonds involved in the linkage of these mitogens to their receptors, we have found that the linkages are not the same. When ^{125}I-TH or ^{125}I-EGF were incubated with HF cells fol-

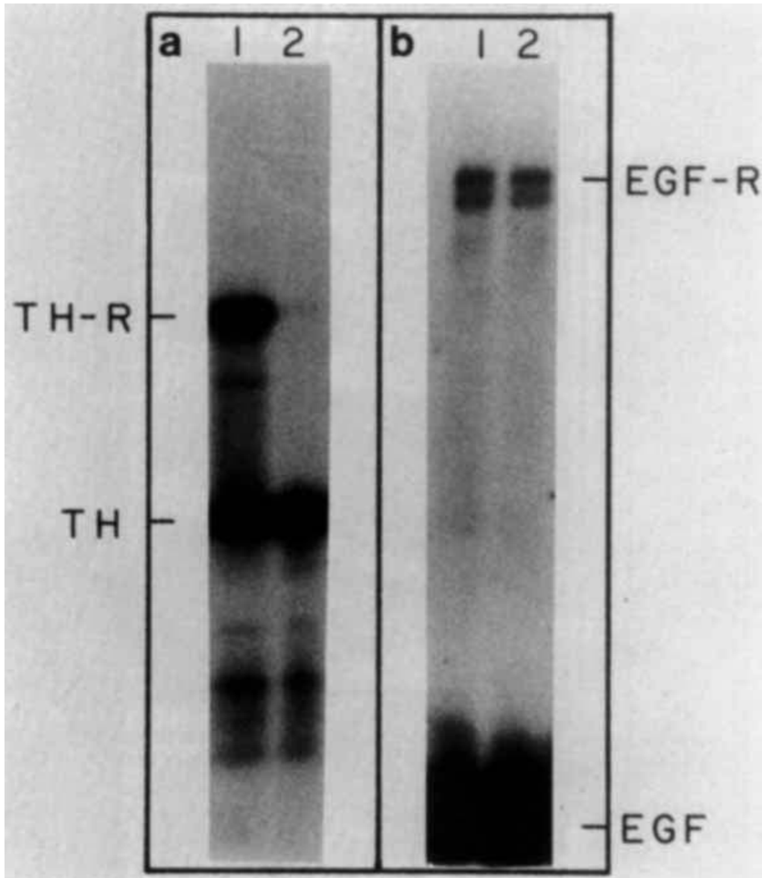

Fig. 6. Stability of TH-R and EGF-R in pH 12 buffer. Confluent HF cultures were incubated in 200 ng/ml ^{125}I-TH (panel a) or 40 ng/ml ^{125}I-EGF (panel b) for 60 min at 37°C. After heating, solubilized samples were dialyzed against 0.1% (w/v) SDS and 50 mM NaCl, then the pH was raised to 12 for 30 min. Samples were then dialyzed against 0.1% SDS, 50 mM Tris-Cl (pH 7.0), 50 mM NaCl before electrophoresis. Lane 1, control, no pH 12 treatment. Lane 2, pH 12 treatment.

lowed by solubilization, electrophoresis, and autoradiography, bands corresponding to TH-R and TH (Fig. 6a, lane 1) and EGF-R and EGF (Fig. 6b, lane 1) were observed. If, however, the pH of the solubilized samples were raised to 12 and then returned to 7.0 before electrophoresis, TH-R disappeared (Fig. 6a, lane 2), whereas EGF-R appeared unaffected (Fig. 6b, lane 2). This strongly suggests that a different type of linkage is involved between these mitogens and their receptors.

Further, it appeared that all of the ^{125}I radioactivity in TH-R was associated with TH, for after disruption by pH 12 virtually all of the radioactivity associated with TH-R migrated with ^{125}I-TH. Figure 6a shows that pH 12 treatment also released ^{125}I-TH from a minor component that was smaller than TH-R. It is not clear whether this component resulted from degradation of TH-R or from linkage of ^{125}I-TH to a different cellular component. The disruption of TH-R by pH 12 suggested that the linkage between TH and its receptor involved an ester bond, based on previous studies of the linkage between TH and antithrombin III [20].

DISCUSSION

We have previously reported that ^{125}I-TH upon incubation with HF cells [1] or 2° mouse embryo cells [17] formed a complex with its receptor. TH-R was resistant to boiling in 3% SDS and 1% 2-mercaptoethanol and was derived exclusively from a pool of ^{125}I-TH that had become specifically bound to the cell surface. Up to 60% of the specifically bound ^{125}I-TH became linked to cellular receptors in HF cells. TH-R formation took place at the cell surface and was an extremely rapid process that preceded by several minutes the accumulation of ^{125}I-TH that was bound but unlinked. These observations suggested that TH-R formation was an important event following ^{125}I-TH binding to cell receptors.

Murine [2] and human [1] cells have also been shown to form a complex between ^{125}I-EGF and its cell surface receptor. Like TH-R, EGF-R was resistant to boiling in SDS and 2-mercaptoethanol, was formed at the cell surface, and was a result of specific binding of ^{125}I-EGF. The amount of EGF-R formed depended upon the concentration of ^{125}I-EGF in the medium and represented a fairly constant portion (6–9%) of the specifically bound ^{125}I-EGF in HF cells.

It appears that the linkages between these mitogens and their receptors are probably covalent, since they survive boiling in SDS and 2-mercaptoethanol. However, the linkages are different, since TH-R but not EGF-R is disrupted by raising the pH to 12. It is noteworthy that the linkage formed between TH and antithrombin III involves an ester bond and is also disrupted at pH 12. Thus, it appears that the linkage between TH and its receptor involved an ester bond. The chemical nature of the linkage between EGF and its receptor is unknown.

EGF-R formation has been reported in HF cells [1], 3T3 cells, SV40-transformed 3T3 cells, A-431 cells (a human tumor cell line), and human placental membranes [2]. Where reported, the molecular weight of EGF-R in the different cell types appeared to be very similar. In the present paper we have shown that TH-R was formed in a variety of cell types that range from avian to rodent and human origin and include normal and malignant cells. The molecular weights of TH-R in these different cells, although very similar, exhibited species-specific differences. Whether these differences are due to amino acid structure, carbohydrate content (if the receptor is a glycoprotein), or modification of TH-R remains to be determined.

Another difference among cell types was the contribution to specifically bound ^{125}I-TH that was due to TH-R. We consistently found that for HF and HT1080 cells TH-R represented a greater proportion of the specifically bound ^{125}I-TH than was seen for 2°CE, D98, or CHL cells. Since HF, HT1080, and D98 cells were all of human origin, this difference, unlike the molecular-weight differences, did not correlate with the species of origin. To determine if there was a relationship between TH-R formation and the ability to respond to TH, we also examined the mitogenic effect of TH on these cells. We previously reported that addition of TH to nondividing cultures of 2°CE [16] and HF cells [21] resulted in cell number increases of 60–70% and 20%, respectively. When CHL and HT1080 cells were treated with TH under similar conditions, CHL cultures increased in cell number by as much as 70%, while HT1080 cultures only increased by 20%. Thus, HF and HT1080 cells which formed greater amounts of TH-R did not respond as well mitogenically to TH as did 2°CE and CHL cells, which formed less TH-R. This suggested an inverse relationship between TH-R and mitogenesis. However, our experiments only measured the amount of TH-R present after 1 h of incubation with ^{125}I-TH. If the rate of degradation relative to rate of formation of TH-R were higher in 2°CE and CHL cells than in HF and HT1080 cells, this might account for the build-up of TH-R in HF and HT1080 cells compared to 2°CE and CHL cells. We are presently investigating this possibility.

Previously only human fibroblasts [16, 22] and 2°CE and 2° mouse embryo cells [16] were known to respond mitogenically to thrombin in the absence of serum or other growth factors. Since these were cell strains and thus had a finite life-span, it was not possible to select stable clones that differed in their mitogenic response to TH. In this paper we have reported a mitogenic response to TH by CHL cells in serum-free medium. It should now be possible to produce 1) clones of mitogenically responsive and nonresponsive cells, 2) clones that differ in the number of TH receptors, and 3) clones that have different rates of formation, internalization, or degradation of TH-R. By studying these kinds of variants, it should be possible to obtain a clearer understanding of the role of TH receptors and TH-R in thrombin-stimulated cell division.

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