Receptor-bound Somatostatin and Epidermal Growth Factor Are Processed Differently in GH4C1 Rat Pituitary Cells

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Abstract. GH4C1 cells, a clonal strain of rat pituitary tumor cells, have high-affinity, functional receptors for the inhibitory hypothalamic peptide somatostatin (SRIF) and for epidermal growth factor (EGF). In this study we have examined the events that follow the initial binding of SRIF to its specific plasma membrane receptors in GH4C1 cells and have compared the processing of receptor-bound SRIF with that of EGF. When cells were incubated with [125I-Tyr]SRIF at temperatures ranging from 4 to 37°C, >80% of the specifically bound peptide was removed by extraction with 0.2 M acetic acid, 0.5 M NaCl, pH 2.5. In contrast, the subcellular distribution of receptor-bound [125I]-EGF was temperature dependent. Whereas >95% of specifically bound [125I]-EGF was removed by acid treatment after a 4°C binding incubation, <10% was removed when the binding reaction was performed at 22 or 37°C. In pulse-chase experiments, receptor-bound [125I]-EGF was transferred from an acid-sensitive to an acid-resistant compartment with a half-time of 2 min at 37°C. In contrast, the small amount of [125I-Tyr]SRIF that was resistant to acid treatment did not increase during a 2-h chase incubation at 37°C. Chromatographic analysis of the radioactivity released from cells during dissociation incubations at 37°C showed that >90% of prebound [125I]-EGF was released as [125I]-tyrosine, whereas prebound [125I-Tyr]SRIF was released as a mixture of intact peptide (55%) and [125I]-tyrosine (45%). Neither chloroquine (0.1 mM), ammonium chloride (20 mM), nor leupeptin (0.1 mg/ml) increased the amount of [125I-Tyr]SRIF bound to cells at 37°C. Furthermore, chloroquine and leupeptin did not alter the rate of dissociation or degradation of prebound [125I-Tyr]SRIF. In contrast, these inhibitors increased the amount of cell-associated [125I]-EGF during 37°C binding incubations and decreased the subsequent rate of release of [125I]-tyrosine.

The results presented indicate that, as in other cell types, EGF underwent rapid receptor-mediated endocytosis in GH4C1 cells and was subsequently degraded in lysosomes. In contrast, SRIF remained at the cell surface for several hours although it elicits its biological effects within minutes. Furthermore, a constant fraction of the receptor-bound [125I-Tyr]SRIF was degraded at the cell surface before dissociation. Therefore, after initial binding of [125I-Tyr]SRIF and [125I]-EGF to their specific membrane receptors, these peptides are processed very differently in GH4C1 cells.

Somatostatin (SRIF) is a 14 amino acid peptide that inhibits secretion by a large variety of pituitary, pancreatic, and gastrointestinal target cells (28). The best-documented physiological function for SRIF is in the control of growth hormone secretion by the pituitary gland (28). GH4C1 cells, a clonal strain of rat pituitary tumor cells that synthesize and secrete both growth hormone and prolactin, provide a homogeneous population of pituitary target cells for studies of SRIF action (33, 40). These cells have specific, high-affinity membrane receptors for SRIF that mediate the inhibitory effects of the peptide on hormone secretion (32, 33, 35). Inhibition of secretagogue-stimulated hormone release by SRIF results from its inhibition of secretagogue-stimulated adenylate cyclase activity (19) and cyclic AMP production (9, 10). In addition, SRIF inhibition of basal hormone secretion may result from a reduction in intracellular calcium levels (10, 20, 31). However, although SRIF receptors have been characterized in GH4C1 cells, as well as in other cell types, little is known about the processing of receptor-bound SRIF after the initial binding event at the plasma membrane.

All peptide ligands examined to date have been shown to interact first with membrane receptors on the cell surface. Thereafter many peptides are rapidly internalized by receptor-mediated endocytosis and subsequently degraded in lysosomes (1, 14, 23, 25). Therefore, the importance of this internalization process in eliciting a specific biological response and in regulating target cell sensitivity to peptide hormones and growth factors is of general interest. In the

Abbreviations used in this paper: EGF, epidermal growth factor; F10-bh, Ham's F10 medium supplemented with 5 mg/ml lactalbumin hydrolysate; SRIF, somatostatin; TLCK, Np-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.
present study we have examined the processing events that
follow the surface binding of [\( ^{125} \text{I}-\text{Ty}r \)]SRIF in GH \(_{3} \)C \(_{1}\) cells. Because [\( ^{125} \text{I}-\text{Ty}r \)]SRIF appeared to be degraded by an unu-
sual, nonlysosomal mechanism, we examined the processing of
receptor-bound [\( ^{125} \text{I}-\text{Ty}r \)]SRIF. In contrast, under the same conditions, [\( ^{125} \text{I}-\text{Ty}r \)]SRIF
goes receptor-mediated endocytosis and lysosomal degrada-
tion. Unlike its mitogenic effect in most other target cells, reduces
epidermal growth factor (EGF) stimulates prolactin release
experiments. We have previously shown that in GH \(_{3} \)C \(_{1}\) cells
epidermal growth factor (EGF) stimulates prolactin release
in contrast, under the same conditions, [\( ^{125} \text{I}-\text{Ty}r \)]SRIF
is not internalized, and lysosomal proteases do not play a role
in receptor-mediated [\( ^{125} \text{I}-\text{Ty}r \)]SRIF degradation. A prelimi-
nary report of some of these results has been presented (27).

**Materials and Methods**

**Materials**

Materials were obtained from the following sources: synthetic SRIF, [\( \text{[Ty}r \_4 \text{]} \)SRIF; thyrotropin-releasing hormone, and bombesin from Bachem, Torrance, CA; EGF from ICN Biomedicals, Inc., Cambrige, MA; insulin, chloroquine, leupeptin, tyrosine, \( 1,1'-\text{tosylamide-2-phenylethy}
loromethyl ketone (TPCK), \( \text{Np}-\text{tosyl-l-lysine chloromethyl}
ketone (TLCK), iodoacetamide, phenylmeth-
ylethyl sulfonate (Fmeh), bacitracin, soybean trypsin inhibitor, porcine plas-
minogen, activated charcoal, T70 dextran, and \( 2,2'-\text{ethanediyloxy}
ethylene (Heps) from Sigma Chemical Co., St. Louis, MO; chloromercurobenzene from Aldrich Chemical Co., Milwaukee, WI; chlor-
mmerine T from Eastman Kodak Co., Rochester, NY; [\( ^{125} \text{I} \)H(N)]sucrose (10.8 Ci/mmol) and 2-deoxy-[\( ^{1,2} \text{H} \)]glucose (37 Ci/mmol)
from New England Nuclear, Boston, MA; Na\(^{25} \)I (17 Ci/mg) and lactalbumin hydrolysate from I.C.N. Nutritional Biochemicals, Cleveland, OH; Sep-Pak C\(_{18}\) cartridges from Waters Associates, Milford, MA; Ham's F10 culture medium and sera from Gibco, Grand Island, NY; and culture plates from Falcon Labware, Oxnard, CA. Phosphoramidon was the kind gift of Dr. A. Goldberg, Harvard Medical School, Boston, MA.

**Cell Culture**

The properties of the GH \(_{3} \)C \(_{1}\) clonal rat pituitary tumor cell strain and the
Materials of its culture have been described previously (32, 33, 40). Experiments
were performed using 35-mm dishes inoculated at a density of 2 × 10\(^{3}\) cells/
dish from a single donor cell suspension and grown under identical conditions.
In 5% CO\(_{2}/95%\) air at 37°C. The culture medium was replaced every 3-4 d,
and experiments were conducted 1.5-2 wk after plating.

**Radioiodination of Peptides and Tyrosine**

[\( ^{125} \text{I}-\text{Ty}r \)]SRIF was prepared by chloramine T iodination as described previ-
ously (32). The reaction mixture was purified on a Sephadex G-25 column
eluted with 20 mM Hepes at pH 7.4 (Hepes-Fl0-1h). The binding of both [\( ^{125} \text{I}-\text{Ty}r \)]SRIF and [\( ^{125} \text{I} \)EGF binding were measured in the presence
of 100 nM SRIF and 10 nM EGF, respectively, and composed 25% of total
[\( ^{125} \text{I} \)EGF binding and 5% of total [\( ^{125} \text{I} \)EGF binding. Values for specific
binding are reported in all experiments unless otherwise specified. The standard
error for specific binding was calculated by taking the square root of the sum
of the squares of the standard errors for total and nonspecific binding.

**Measurement of 2-Deoxy-[\( ^{1,2} \text{H} \)]-Glucose and [\( \text{Fructose-1,2-3H(N)} \)Sucrose Uptake**

Deoxyxglucose and sucrose uptake were measured under the same incubation
conditions as peptide binding. After equilibration at 37°C, cells were incubated
with 3 \( \mu \)Ci/ml of 2-deoxy-[\( ^{1,2} \text{H} \)]glucose or 5 \( \mu \)Ci/ml of [\( \text{Fructose-1,2-3H(N)} \)sucrose for 60 min. The cells were subsequently rinsed rapidly with cold
saline, incubated for an additional 10 min at 37°C to allow surface-bound
deoxyxglucose or sucrose to dissociate, and then dissolved in 0.8 ml of 1%
Nonidet P-40. The cell-associated radioactivity was measured in 14 ml Aqualos
with a Beckman LS7000 scintillation counter at a counting efficiency of 30%.

**Extraction of Cell-associated Radioactivity with Acid/Salt**

The procedure of Haigler et al. (15) was used to determine the cellular
distribution of specifically bound peptides. After the appropriate incubations
with radiolabeled peptide, cells were washed with cold saline and treated with
1.0 ml of 4°C 0.2 M acetic acid, 0.5 M NaCl, ph 2.5 (acid/salt). After 3-5 min
at 4°C, the extract was collected, the cells were rinsed with an additional 1 ml
of acid/salt solution, and the two extracts were combined. Cells were then
dissolved in 0.8 ml of 1 N NaOH. The radioactivity in both the acid/salt extract
and the base solubilized cell residue was measured. All values were corrected
to represent specific binding as described above.

**Measurement of [\( ^{125} \text{I}-\text{Ty}r \)]SRIF Binding to Membranes**

GH \(_{3} \)C \(_{1}\) cell membranes were prepared by the procedure of Koch and Schon-
brunn (19). Freshly thawed cell membranes were washed twice and re suspended
in cold Tris buffer (50 mM Tris-HCl, 2 mM MgCl\(_{2}\), 2 mM EDTA, pH 7.6) at
a protein concentration of ~1 mg/ml. The membranes were Teflon on glass
homogenized at 4°C, and an equal volume of cold 50 mM Tris-HCl, 12 mM
MgCl\(_{2}\), 2 mM EDTA, 25% BSA, 4 U/ml bacitracin, ph 7.6, was added. The
binding reaction was carried out in 1 ml of this buffer containing ~150 pg
membrane protein and 100,000 cpm [\( ^{125} \text{I} \)Ty]
SRIF. After 90 min at 37°C, the membranes were diluted with 3 vol 50 mM Tris-HCl, 7 mM MgCl\(_{2}\), 2 mM EDTA, 2 U/ml bacitracin, ph 7.6 (binding buffer) containing 1% BSA and
collected by centrifuging at 40,000 g for 10 min at 4°C. The supernatants
were removed, and the amount of radioactivity in the membrane pellet was deter-
mimed. Specific [\( ^{125} \text{I} \)Ty]SRIF binding was calculated as described for experi-
ments with intact cells and represented 50-70% of total membrane binding.

For experiments examining the dissociation of membrane-bound [\( ^{125} \text{I} \)Ty-
SRIF], the membranes were washed twice by centrifugation at 10,000 g for
25 min at 4°C and then were incubated at 37°C in binding buffer containing
0.05% BSA for the indicated period. At the end of this dissociation incubation
the membranes were centrifuged at 40,000 g for 10 min at 4°C, and the amount
of radioactivity in the supernatant and in the pellet was determined.

**Analysis of Radiolabeled Peptide Degradation**

Degradation of [\( ^{125} \text{I} \)EGF was assessed by chromatography on Bio-Gel P2
columns eluted with 0.05 M NaPO\(_{4}\), 0.075 M NaCl, 0.1% BSA, ph 7.5. [\( ^{12} \text{I} \)EGF and [\( ^{12} \text{I} \)tyrosine standards were run under the same conditions as exper-
imental samples and eluted at single peaks.

Degradation of [\( ^{12} \text{I} \)Ty]SRIF was determined using a modification of the
method of Bohlen et al. (3). Samples were applied to Sep-Pak C\(_{18}\) reversed
phase cartridges prewashed with 10 ml methanol and 10 ml water. Na\(^{25} \)I was
Measurement of [125I-Tyr]SRIF Binding to Cytosolic Proteins

The procedure of Rey et al. (29, 30) was adapted for the detection of cytosolic [125I-Tyr]SRIF binding proteins. GH4C1 cells were homogenized in 250 mM sucrose, 0.1 mg/ml soybean trypsin inhibitor, 10 mM Tris-HCl, pH 7.4. The cell homogenate was centrifuged at 100,000 g for 60 min at 4°C to remove all particulate material. Aliquots of the supernatant (0.25–0.6 mg protein) were incubated with [125I-Tyr]SRIF (50,000 cpm) for 15 min at 22°C in total volume of 0.5 ml. One ml of 0.25% charcoal, 0.025% T70 dextran, 0.5% BSA in 50 mM Tris-HCl, pH 7.4, was then added, and the suspension was centrifuged at 2,500 g for 10 min at 4°C. This charcoal/dextran adsorption procedure removes 99% of unbound [125I-Tyr]SRIF from solution. The amount of [125I-Tyr]SRIF and [125I]-tyrosine standards were carried through the same protocol as experimental samples and eluted as single peaks.

Measurement of Cell Number and Protein

Cell number was determined as described by Schonbrunn et al. (34). Experiments were performed at a cell density of 1.5–2 x 10^6 cells/35 mm dish. Cell protein was measured by the Peterson modification (26) of the Lowry method using bovine albumin as a standard.

Results

Cellular Distribution of Receptor-bound Peptides

Both morphological and biochemical studies have shown that fibroblasts internalize EGF by receptor-mediated endocytosis within minutes of receptor occupancy at physiologic temperatures (1, 4, 14, 15, 23, 25). To determine whether receptor-bound SRIF was processed by a similar pathway, we have used an acid extraction protocol originally developed by Haigler et al. for the rapid quantitation of surface bound and internalized [125I]-EGF (15). We have previously used this protocol to demonstrate that in GH4C1 cells, as in fibroblasts, receptor-bound EGF is transformed from an acid-extractable to an acid-resistant state within 10 min at 37°C (42). The experiment in Table I was carried out to determine the cellular distribution of [125I-Tyr]SRIF bound at different temperatures.

GH4C1 cells were incubated with [125I-Tyr]SRIF at various temperatures and then treated with cold acid/salt as described in Materials and Methods (Table I). Unexpectedly, 86 ± 4% of the specifically bound [125I-Tyr]SRIF was acid extractable regardless of the temperature of the binding incubation. In contrast, the distribution of receptor-bound [125I]-EGF after similar binding incubations was markedly temperature dependent: the fraction of specifically bound [125I]-EGF that was dissociated by acid after 2-h binding incubations at either 4 or 37°C were 97 and 6%, respectively.

To evaluate the extent of cellular leakage caused by acid treatment, we measured the effect of the acid extraction procedure on cell-associated 2-deoxy-[1,2-3H]-glucose. This glucose analogue is phosphorylated intracellularly and provides a useful marker for cytoplasmic constituents. After a 60-min binding incubation at 37°C, exposure of cells to cold 0.2 M acetic acid/0.5 M NaCl, pH 2.5, for 3 min removed >80% of specifically bound [125I-Tyr]SRIF. However, in the same experiment, <10% of cell-associated 2-deoxyglucose was removed by acid extraction (data not shown). Furthermore, extraction periods of 30 s to 15 min gave identical results. Therefore, treatment of GH4C1 cells with an acid solution dissociates most of the specifically bound [125I-Tyr]SRIF without causing significant leakage of cytoplasmic contents.

The results in Table I indicated that if receptor-mediated internalization of [125I-Tyr]SRIF occurred, its rate was much slower than that for [125I]-EGF (42). To examine this difference further, we determined the cellular distribution of bound peptides after different periods of binding at 37°C (Fig. 1). After 2 min at 37°C, 80% of the specifically bound [125I]-EGF was removed by acid treatment (Fig. 1, inset). However, the fraction of specifically bound [125I]-EGF that was resistant to acid extraction increased rapidly thereafter, and by 60 min <10% of the specifically bound radioactivity could be released (Fig. 1, top). In contrast, 82 ± 1% of the specifically bound [125I-Tyr]SRIF was removed by acid treatment at all time points examined (Fig. 1, bottom). The acid-resistant [125I-Tyr]SRIF binding could not be accounted for by bulk pinocytosis of the binding medium. Measurement of [fructose-1,6(NH)H]sucrose uptake showed the rate of bulk pinocytosis at 37°C to be 1.1 ± 0.1 (n = 2) nM/min per 10^6 cells. This rate of pinocytosis could account for the internalization of <20 cpm of [125I-Tyr]SRIF during the 60-min binding incubation.

The low steady state level of intracellular [125I-Tyr]SRIF could result from ligand internalization being followed rapidly by degradation and extrusion of radiolabeled degradation products. If this were the case, radioactivity would not accumulate intracellularly. Therefore, to examine directly the fate of surface-bound [125I-Tyr]SRIF and [125I]-EGF, we carried out pulse-chase experiments in which the redistribution of receptor-bound peptides between acid-sensitive and resistant compartments was monitored (Fig. 2). The binding of [125I]-EGF was carried out at 4°C so that this peptide would remain at the cell surface. However, since the amount of [125I-Tyr]SRIF bound to cells at 4°C was substantially less than that bound at higher temperatures, and the fraction of the bound radioactivity that was acid extractable was independent of the

Table I. Effect of Binding Temperature on the Distribution of [125I-Tyr]SRIF

| Binding incubation | Specific binding | A/S resistant |
|--------------------|------------------|---------------|
|                    | Total            | A/S resistant | %   |
| 4°C 5 h            | 2,529 ± 738      | 200 ± 156     | 8   |
| 22°C 3 h           | 10,510 ± 654     | 1,437 ± 52    | 14  |
| 37°C 1 h           | 6,712 ± 580      | 1,341 ± 156   | 20  |

GH4C1 cells were incubated with [125I-Tyr]SRIF (102,000 cpm/ml, 35 PM) under the conditions shown. The amount of specifically bound peptide was then determined before (total) and after acid/salt (A/S resistant) treatment of the cells with 0.2 M acetic acid/0.5 M NaCl, pH 2.5, for 2-3 min at 4°C.

* Mean ± SEM of triplicate determinations.
temperature of the binding incubation (Table I), [125I-Tyr]*SRIF binding was done at 37°C. After the binding incubations, 37°C medium without radiolabeled peptides was added to cells, and the distribution of receptor-bound peptides was determined as a function of time (Fig. 2).

Receptor-bound 125I-EGF was rapidly transformed from an acid-extractable to an acid-resistant compartment at 37°C (t₀ = 2 min). In contrast, the fraction of receptor-bound [125I-Tyr]*SRIF present in an acid-resistant state remained at or below its initial value of 20% throughout a 90-min chase incubation at 37°C. Although the amount of acid-extractable [125I-Tyr]*SRIF decreased during this period (t₀ = 60 min), this decrease was due to dissociation of the peptide into the medium, not to its translocation into an acid-resistant compartment. The addition of a saturating concentration of SRIF (100 nM) to the chase medium did not alter the distribution of receptor-bound [125I-Tyr]*SRIF (data not shown), demonstrating that the level of receptor occupancy did not influence the processing of receptor-bound ligand.

In summary, surface bound [125I-Tyr]*SRIF was not redistributed in pulse-chase experiments. In addition, the fraction of receptor-bound [125I-Tyr]*SRIF that was acid-resistant was always small (<20%) and did not vary with either the temperature or the duration of the binding incubation. These data argue strongly for the conclusion that the 10 to 20% of [125I-Tyr]*SRIF binding that was acid resistant results from incomplete dissociation under the conditions of the extraction procedure. Therefore, our results indicate that in GH4C1 cells, receptor-bound 125I-EGF is rapidly internalized whereas [125I-Tyr]*SRIF is not.

In addition to EGF, three other peptides also appear to undergo rapid receptor-mediated endocytosis in GH4C1 cells: thyrotropin releasing hormone (17), bombesin (42), and insulin (7). However, the addition of saturating concentrations of these peptides to the chase medium did not alter the fraction of receptor-bound [125I-Tyr]*SRIF that was dissociated by acid after either 30- or 60-min incubations at 37°C (data not shown). Thus, simultaneous, rapid endocytosis of heterologous peptides do not affect the cellular distribution of receptor-bound [125I-Tyr]*SRIF.

Degradation of Receptor-bound Peptides

The lack of internalization of receptor-bound [125I-Tyr]*SRIF...
types: rapid internalization of the peptide is followed by consistent with the fate of receptor-bound \(^{125}\)I-EGF in other cell >90% \(^{125}\)I-tyrosine (data not shown). These results are con-
a subsequent 60-min incubation at 37°C chromatographed as
as -50% intact peptide and -50% \(^{125}\)I-tyrosine at all time
points examined. In contrast, when cells were incubated with
as -50% intact peptide and 50% \(^{125}\)I-tyrosine (Table II). This degradation was not
due to instability of the peptide in the medium since \(^{125}\)I-
Tyr'\]SRIF and possible intermediate peptide degra-
dation products (Fig. 3).

After incubation of \(^{125}\)I-Tyr'\]SRIF with \(GH_4C_1\) cells for 60
min at 37°C, the specifically bound radioactivity extracted
from cells with acid/salt co-eluted with intact \(^{125}\)I-Tyr'\]SRIF
both on minicolumns (Table II) and high-performance liquid
chromatography (Fig. 3 C). Therefore, \(^{125}\)I-Tyr'\]SRIF specifically
bound at the surface of cells represents intact peptide.
In contrast, the specifically bound radioactivity released into
the medium during a subsequent 60-min incubation at 37°C chromatographed as ~50% intact peptide and 50% \(^{125}\)I-tyrosine (Table II and Fig. 3, A and B). This degradation was not
due to instability of the peptide in the medium since \(^{125}\)I-
Tyr'\]SRIF incubated in F10-lh for 60 min under the same
conditions remained 95% intact (Table II). Furthermore, 90% of the nonspecifically bound \(^{125}\)I-Tyr'\]SRIF dissociated as
intact peptide (Table II), indicating that the degradation must
be a receptor-mediated process.

We next measured the rates of release of both intact \(^{125}\)I-
Tyr'\]SRIF and \(^{125}\)I-tyrosine from cells to determine whether
radioactivity was released primarily as intact peptide early and as degradation products later. The results shown in Fig. 4 demonstrate that the rates of release of intact \(^{125}\)I-
Tyr'\]SRIF and \(^{125}\)I-tyrosine from cellular receptors were in-
distinguishable: receptor-bound \(^{125}\)I-Tyr'\]SRIF was released as ~50% intact peptide and ~50% \(^{125}\)I-tyrosine at all time
points examined. In contrast, when cells were incubated with
\(^{125}\)I-EGF for 60 min at 37°C, the radioactivity released during a subsequent 60-min incubation at 37°C chromatographed as >90% \(^{125}\)I-tyrosine (data not shown). These results are con-
sistent with the fate of receptor-bound \(^{125}\)I-EGF in other cell
types: rapid internalization of the peptide is followed by

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**Table II. Degradation of Receptor-bound \(^{125}\)I-Tyr'\]SRIF**

| Sample                  | \(^{125}\)I | \(^{125}\)I % Total | \(^{125}\)I Fraction I | \(^{125}\)I Fraction II | \(^{125}\)I Fraction III |
|------------------------|-----------|-----------------|----------------------|------------------------|--------------------------|
| Standards              | 43,040 ± 210 | 2               | 2                    | 97                     |
| \(^{125}\)I-Tyr'\]SRIF  | 30,530 ± 180 | 5               | 5                    | 5                      |
| Binding medium         | 44,000 ± 210 | 1               |                       | 95                     |
| Cell extract           | 7,490 ± 363  | 0               | 1                    | 99                     |
| Dissociation medium    | 4,700 ± 350  | 2               | 46                   | 53                     |
| Specific               | 2,190 ± 270  | 0               | 11                   | 89                     |

\(GH_4C_1\) cells were incubated at 37°C with \(^{125}\)I-Tyr'\]SRIF (150,000 cpm/ml, 51 pM) in the presence or absence of 100 nM SRIF. After 60 min, the binding medium was removed, and the cells were rapidly rinsed in 4°C saline before being divided into two groups. One group was immediately extracted with 1 ml 0.2 M acetic acid/0.5 M NaCl, pH 2.5, for 3 min at 4°C (Cell extract). The other group was incubated for an additional 60 min at 37°C in 1.0 ml fresh F10-1h medium to allow dissociation of cell-associated radioactivity (dissociation medium). Samples and standards were analyzed by chromatography on reverse-phase minicolumns as described in Materials and Methods. Fractions I, II, and III represent the radioactivity eluted with 0.1% trifluoroacetic acid, in 0, 25, and 80% methanol, respectively. Recovery of radioactivity from the columns was >95% for all samples. Errors were ± 2% for triplicate determinations.

* Mean ± SEM of duplicate determinations.

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**Figure 3. Identification of the degradation products of receptor-bound \(^{125}\)I-Tyr'\]SRIF by HPLC.** \(GH_4C_1\) cells were incubated with \(^{125}\)I-
Tyr'\]SRIF for 60 min at 37°C. After the binding incubation, the cells were rapidly rinsed in 4°C saline, incubated for 5 min at 37°C to allow >75% of the nonspecifically bound \(^{125}\)I-Tyr'\]SRIF to dissociate, and then divided into two groups. One group was immediately extracted with 0.2 M acetic acid/0.5 M NaCl (cell extract). The other group was incubated for an additional 60 min at 37°C in fresh medium to allow dissociation of cell-associated radioactivity (release medium). Samples were first purified by stepwise elution on reverse-phase minicolumns and then analyzed by reverse-phase high-performance liquid chromatography as described in Materials and Methods. A and B show the elution profiles of the release media fractions eluted from reverse-phase minicolumns with 25% methanol/0.1% trifluoroacetic acid (fraction II, 7,100 cpm) and 80% methanol/0.1% trifluoroacetic acid (fraction III, 7,000 cpm), respectively. C represents the elution profile of the cell extract fraction eluted from Sep-Pak minicolumns with 80% methanol/0.1% trifluoroacetic acid (fraction III, 24,000 cpm). The retention times of \(^{125}\)I-SRIF and \(^{125}\)I-tyrosine standards are shown by arrows. Recovery for all samples was >85%.
degradation and subsequent extrusion of 125I-tyrosine from cells (1, 14, 23, 25).

The results presented demonstrate that [125I-Tyr]SRIF is degraded upon dissociation from cellular receptors without prior internalization. Therefore, in marked contrast to the lysosomal degradation previously reported for 125I-EGF and other internalized peptide ligands, receptor-mediated degradation of [125I-Tyr]SRIF is more likely to occur at the plasma membrane than in lysosomes.

**Effect of Lysosomal Inhibitors on Peptide Binding and Processing**

To evaluate further the role of lysosomal enzymes in the degradation of receptor-bound peptides in GH4C1 cells we determined the effects of the lysosomal inhibitors chloroquine, ammonium chloride, and leupeptin on the binding of 125I-EGF and [125I-Tyr]SRIF and the subsequent release of their degradation products. Chloroquine and ammonium chloride inhibit lysosomal proteolysis by reducing the transmembrane pH gradient of lysosomes. Leupeptin is a specific inhibitor of thiol proteases, including cathepsin B. The results in Fig. 5 and Table III show that chloroquine and leupeptin increased the amount of cell-associated 125I-EGF during prolonged 37°C binding incubations. However, neither inhibitor

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**Table III. Effect of Leupeptin and Chloroquine on [125I-Tyr]SRIF Processing**

| Chase incubation | Treatment | [125I-Tyr]SRIF bound | % A/S resistant | % Intact peptide |
|------------------|-----------|----------------------|----------------|-----------------|
| Time min         |           | Total cpm*          | A/S resistant cpm* |                |
| 0                | Control   | 10,677 ± 463        | 1,675 ± 98      | 16              |
|                  | Leupeptin | 9,346 ± 92          | 1,472 ± 8       | 16              |
|                  | Chloroquine | 5,215 ± 131        | 952 ± 43        | 18              |
| 30               | Control   | 6,150 ± 151         | 1,144 ± 44      | 9               |
|                  | Leupeptin | 5,537 ± 28          | 1,031 ± 19      | 9               |
|                  | Chloroquine | 3,491 ± 76         | 800 ± 27        | 23              |
| 60               | Control   | 3,110 ± 301         | 602 ± 34        | 9               |
|                  | Leupeptin | 3,385 ± 212         | 777 ± 85        | 23              |
|                  | Chloroquine | 1,725 ± 146     | 505 ± 59        | 29              |

GH4C1 cells were preincubated at 37°C with 0.1 mg/ml leupeptin for 60 min or 0.1 mM chloroquine for 30 min. [125I-Tyr]SRIF (150,000 cpm/ml, 51 pM) was then added, and the incubations were continued for 60 min at 37°C. The percent intact peptide was calculated from the ratio of the amount of radioactivity eluted from the columns with 80% methanol, 0.1% trifluoroacetic acid to the total radioactivity eluted. All values have been corrected for nonspecific binding. * Mean ± SEM of duplicate or triplicate determinations.
increased the amount of \([^{125}\text{I}}\text{-Tyr}^1\]SRIF bound to cells during a 4 to 6 h incubation at 37°C. In fact, chloroquine caused a marked decrease in \([^{125}\text{I}}\text{-Tyr}^1\]SRIF binding at all times examined. However, the effect of chloroquine to decrease \([^{125}\text{I}}\text{-Tyr}^1\]SRIF binding is probably not a result of its lysosomotropic action. When lysosomal function was inhibited with 20 mM ammonium chloride, the amount of \([^{125}\text{I}}\text{-EGF} bound to cells during 4 h at 37°C was increased to 210% of untreated controls. In the same experiment \([^{125}\text{I}}\text{-Tyr}^1\]SRIF binding was unaffected by the ammonium chloride.

The results in Fig. 6 show the effect of chloroquine on the rate of release of radioactivity after the binding of \([^{125}\text{I}}\text{-EGF} and \([^{125}\text{I}}\text{-Tyr}^1\]SRIF to cells. The half-time for the release of \([^{125}\text{I}}\text{-tyrosine derived from receptor-bound \([^{125}\text{I}}\text{-EGF was increased from 45 min in control cells to 150 min in chloro-

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Effect of chloroquine on the release of cell-associated radioactivity. **GH4C1 cells were preincubated for 30 min at 37°C either in F10-1h alone (○) or in the presence of 0.1 mM chloroquine (□). Subsequently, either \([^{125}\text{I}}\text{-EGF (125,000 cpm/ml, 59 pM) or \([^{125}\text{I}}\text{-Tyr}^1\]SRIF (103,000 cpm/ml, 35 pM) was added, and the incubations were continued for 90 min for \([^{125}\text{I}}\text{-EGF and 60 min for \([^{125}\text{I}}\text{-Tyr}^1\]SRIF. After the binding incubation, the cells were rapidly rinsed with 4°C saline, and the dissociation reaction was initiated at \(t = 0\) by the addition of fresh 37°C F10-1h medium in the continued absence or presence of chloroquine. The amount of specifically bound peptide was determined at the indicated times as described in Materials and Methods. Specific binding before the initiation of the dissociation reactions was 7,131 ± 67 cpm (○) and 3,949 ± 325 cpm (□) for \([^{125}\text{I}}\text{-Tyr}^1\]SRIF and 13,810 ± 210 (○) and 15,540 ± 70 (□) cpm for \([^{125}\text{I}}\text{-EGF. Each point represents the mean of duplicate determinations. B. bound.**

**Table IV. Degradation of \([^{125}\text{I}}\text{-Tyr}^1\]SRIF Bound to Membranes**

| Dissociation incubation | \([^{125}\text{I}}\text{ bound | \([^{125}\text{I}}\text{ dissociated | % Intact \([^{125}\text{I}}\text{-Tyr}^1\]SRIF |
|------------------------|-----------------|-----------------|-----------------|
| 0                      | 21,449 ± 750    | —               | —               |
| 2 h                    | 15,791 ± 421    | 7,378 ± 50      | 90              |
| 4 h                    | 11,630 ± 742    | 10,576 ± 360    | 87              |
| 4 h + 100 nM SRIF      | 9,695 ± 849     | 10,791 ± 343    | 85              |

**GH4C1 membranes (150 μg/ml) were incubated with \([^{125}\text{I}}\text{-Tyr}^1\]SRIF (342,000 cpm/ml, 120 pM) for 90 min at 37°C in the absence or presence of 100 nM SRIF as described in Materials and Methods. The membranes were then collected by centrifugation, washed twice, and resuspended in 37°C binding buffer containing 0.05% BSA with or without SRIF. After incubating at 37°C with shaking for the indicated amount of time the membranes were centrifuged at 40,000 g for 10 min at 4°C. The nature of the dissociated material present in the supernatants was analyzed on Sep-Pak C18 reverse-phase minicolumns, and the binding of \([^{125}\text{I}}\text{-Tyr}^1\]SRIF to the membranes were determined as described in Materials and Methods. An SEM of triplicate determinations. B. bound.**

To assay directly for membrane proteases that might be responsible for the degradation of receptor-bound \([^{125}\text{I}}\text{-Tyr}^1\]SRIF, we examined the nature of the radioactivity released after binding \([^{125}\text{I}}\text{-Tyr}^1\]SRIF to GH4C1 cell membranes. In contrast to intact cells, 85-90% of the specifically bound radioactivity released from membranes during dissociation incubations of 2-4 h chromatographed as intact \([^{125}\text{I}}\text{-Tyr}^1\]SRIF (Table IV). In addition, the amount of degradation was not affected by 100 nM SRIF. Nonspecifically bound peptide dissociated as 82% intact \([^{125}\text{I}}\text{-Tyr}^1\]SRIF, showing the same small amount of degradation as specifically bound peptide (data not shown). The observation that specific pro tease of receptor-bound peptide did not occur in isolated
membranes indicated either that intact cells were necessary for such degradation or that the proteases involved were inactivated during the process of membrane preparation.

**Effect of Protease Inhibitors on [125I-Tyr]SRIF Degradation**

To characterize further the enzyme(s) responsible for the degradation of receptor-bound [125I-Tyr]SRIF in cells we examined the effects of several protease inhibitors and unlabeled SRIF on peptide degradation. The presence of saturating concentrations of SRIF (100 nM and 0.1 mM) in the dissociation medium had only a slight effect on [125I-Tyr]SRIF degradation (Table V). Therefore this degradation was either catalyzed by a low-affinity enzyme or else receptor-bound peptide was not released into the medium before degradation. Bacitracin, a noncompetitive inhibitor of papain and subtilisin; phenylmethylsulfonyl fluoride, a general irreversible inhibitor of serine proteases; TLCK, an irreversible inhibitor of trypsin-like enzymes; α-g-macroglobulin, which binds and inactivates a wide variety of proteases; and puromycin, an inhibitor of certain aminopeptidases, had the same small effect on receptor-mediated degradation as did 0.1 mM SRIF. However, TPCK, an inhibitor of chymotrypsin-like enzymes; the two sulphydryl reagents iodoacetamide and chloromercuribenzoate; and phosphoramidon, which inhibits metalloendoproteases, substantially inhibited receptor-mediated degradation of [125I-Tyr]SRIF (Table V).

*Table V. Effect of Peptidase Inhibitors on Degradation of [125I-Tyr]SRIF Bound to Cells*

| Treatment            | % Intact [125I-Tyr]-SRIF released |
|----------------------|-----------------------------------|
| Control              | 3,947 ± 358                      |
| SRIF (100 nM)        | 4,362 ± 104                      |
| Bacitracin (0.1 mM)  | 4,293 ± 321                      |
| PMSF (0.1 mM)        | 5,190 ± 501                      |
| TLCK (0.1 mM)        | 5,562 ± 165                      |
| TPCK (0.1 mM)        | 6,357 ± 484                      |
| Iodoacetamide (1.0 mM)| 7,789 ± 105                     |
| Chloromercuribenzoate| 6,834 ± 286                      |
| Exp. 2 (B<sub>max</sub> = 8,064 ± 48) | 6,810 ± 224                  |
| α2-Macroglobulin (50 μg/ml) | 2,217 ± 44           |
| Exp. 3 (B<sub>max</sub> = 9,866 ± 128) | 2,613 ± 124                  |
| Control              | 2,686 ± 130                      |
| Puromycin (0.1 mM)   | 2,034 ± 142                      |
| Phosphoramidon (0.1 mM)| 1,703 ± 45                 |

GH4C<sub>1</sub> cells were incubated for 1 h with [125I-Tyr]SRIF (115.000 cpm/ml, 39 pM) for 60 min at 37°C. After the binding reaction, the cells were washed with 4°C saline and fresh 37°C F10-h containing the indicated compounds was added (t = 0). After 60 min at 37°C, the radioactivity released into the medium was measured and then analyzed by chromatography on reverse-phase minicolumns (see Materials and Methods). The percent intact peptide was calculated from the ratio of the amount of [125I] eluted from the columns with 80% methanol, 0.1% trifluoroacetic acid (fraction II) to the total radioactivity eluted. All values have been corrected for nonspecific binding. For experiment 3, inhibitors were added 30 min before the initiation of the binding incubation. PMSF, phenylmethylsulfonyl fluoride, B, bound.

* Mean ± SEM of triplicate determinations.

**Binding of [125I-Tyr]SRIF to Cytosolic Proteins**

Recent reports have suggested that an intracellular cytosolic receptor for SRIF accounts for most of the [125I-Tyr]SRIF binding in gastric mucosal and pancreatic cells (29, 30). These cytosolic receptors are reported to mediate stimulation of soluble phosphoprotein phosphatases by SRIF (29). However, our results indicate that receptor-bound SRIF is not internalized in GH4C<sub>1</sub> cells. Therefore, following procedures similar to those described by Rey and Lewin (29), we have tried to identify saturable binding sites for [125I-Tyr]SRIF in GH4C<sub>1</sub> cell cytosol. The results in Table VI show that the binding of [125I-Tyr]SRIF to cytosolic proteins was not decreased by 100 nM unlabeled SRIF and therefore must represent binding to a low-affinity site. The lack of high-affinity binding sites for SRIF in GH4C<sub>1</sub> cell cytosol combined with the observation that receptor-bound SRIF remains at the cell surface while it inhibits hormone release indicate that the biological actions of SRIF in GH4C<sub>1</sub> cells are initiated at the plasma membrane.

**Discussion**

In this study we have shown that receptor-bound SRIF and EGF are processed very differently in GH4C<sub>1</sub> cells. Whereas [125I]-EGF underwent rapid receptor-mediated internalization and was subsequently degraded in lysosomes, [125I-Tyr]SRIF remained bound at the cell surface and was degraded in a nonlysosomal compartment. The absence of [125I-Tyr]SRIF internalization is not due to a general defect in GH4C<sub>1</sub> cells: [125I]-EGF is internalized with the same rapid kinetics as in other cell types, and both [125I-Tyr]SRIF and SRIF are bioactively active in GH4C<sub>1</sub> cells under the conditions of the binding incubation (32, 35). In fact, SRIF elicits a maximal inhibitory effect on hormone secretion within 5 min (32). Koch, B. D., and A. Schonbrunn, unpublished observations). The rapid biological action of SRIF combined with the lack of rapid internalization indicate that SRIF must elicit its biological effects at the plasma membrane.

To examine the cellular processing of receptor-bound peptides in GH4C<sub>1</sub> cells, we have used an acid extraction protocol first introduced by Haigler et al. for distinguishing intracellular and extracellular [125I]-EGF (15). This indirect biochemical method for quantitating the extent of internalization of receptor-bound peptides has produced results in excellent agreement with morphological studies using appropriately labeled peptide analogs (1, 14, 23, 25). In fact, pHi-induced dissociation of peptides from their receptors is a physiological
mechanism for intracellular segregation of ligands and their receptors within acidic, prelysosomal vesicles (8, 41).

The kinetics of receptor-mediated internalization, as measured by translocation of bound peptides from an acid-extractable to an acid-resistant compartment, were markedly different for \(^{125}\)I-Tyr\(^{-}\)SRIF and \(^{125}\)I-EGF in GH\(_4\)C1 cells. \(^{125}\)I-EGF was internalized rapidly \((t_{1/2} = 2\) min\), as previously observed in a variety of cell types (1, 4, 14–16, 23, 25, 42). In contrast, under identical conditions, receptor-bound \(^{125}\)I-Tyr\(^{-}\)SRIF was internalized rapidly \((t_{1/2} = 2\) min\), as previously observed in a variety of cell types (1, 4, 14–16, 23, 25, 42). In contrast, under identical conditions, receptor-bound \(^{125}\)I-Tyr\(^{-}\)SRIF was internalized rapidly, whereas \(^{125}\)I-insulin was intact ~25I-insulin, the radioactive material dissociated from receptors consisted of an approximately equal mixture of intact ~25I-EGF. These results indicated that receptor-mediated degradation of ~25I-EGF did not occur in GH\(_4\)C1 cells (13, 38). Whereas >95% of the specifically bound ligand was intact ~25I-EGF, the radioactive material dissociated from receptors consisted of an approximately equal mixture of intact ~25I-EGF and iodotyrosine (13). Degradation was specific for receptor-bound peptide since only intact ~25I-insulin was released from non-specific binding sites. Furthermore, the fraction of receptor-bound ~25I-insulin that was degraded did not vary with receptor occupancies between 1 and 95%, demonstrating that ~25I-insulin degradation was proportional to receptor occupancy (13). Leupeptin did not decrease receptor-mediated ~25I-insulin degradation, nor did it increase the accumulation of cellular ~25I-insulin (38). Although chloroquine did increase cell-associated ~25I-insulin during binding incubations, it had only a small effect on receptor-mediated degradation, and two other lysosomotropic agents, methylamine and NH\(_4\)Cl, did not increase insulin binding (38). Since lysosomotropic agents did not uniformly decrease the degradation of ~25I-insulin or increase its intracellular accumulation, the major degradation pathway for receptor-bound ~25I-insulin in adipocytes was proposed to be nonlysosomal (38).

In GH\(_4\)C1 cells degradation of receptor-bound \(^{125}\)I-Tyr\(^{-}\)SRIF was unaffected by the presence of excess unlabeled SRIF in the dissociation medium. This lack of protection is consistent with the hypothesis that the degrading enzyme or enzymes have low affinity \((K_m > 10^{-5}\) M\) for \(^{125}\)I-Tyr\(^{-}\)SRIF. However, the observation that \(^{125}\)I-Tyr\(^{-}\)SRIF degradation occurred at the very low \(^{125}\)I-Tyr\(^{-}\)SRIF concentrations \((\sim 10^{-13}\) M\) present in the medium when \(^{125}\)I-Tyr\(^{-}\)SRIF dissociates from its receptor makes this explanation unlikely. Alternatively, the receptor itself may mediate the degradation of \(^{125}\)I-Tyr\(^{-}\)SRIF or directly transfer the bound \(^{125}\)I-Tyr\(^{-}\)SRIF to the degrading enzyme(s). The latter mechanisms have also been postulated for the adipocyte insulin receptor but remain to be proven (11, 38).

Membrane proteases have been hypothesized to catalyze the nonlysosomal degradation of insulin in rat adipocytes; however a protease that selectively degrades only receptor-bound ~25I-insulin has not been identified (11, 38). Similarly, we could not identify specific degradation of receptor-bound \(^{125}\)I-Tyr\(^{-}\)SRIF in GH\(_4\)C1 membranes. Although receptor-mediated degradation of ~25I-insulin in adipocytes and \(^{125}\)I-Tyr\(^{-}\)SRIF in GH\(_4\)C1 cells appear to occur by similar mechanisms, there are some significant differences between the two systems. In adipocytes, intact insulin was released somewhat more rapidly than iodotyrosine (13, 24). In fact, the increasing degradation of receptor-bound insulin observed with time correlated with the conversion of the insulin receptor from a rapidly to a slowly dissociating state (24). In contrast, after \(^{125}\)I-Tyr\(^{-}\)SRIF binding to GH\(_4\)C1 cells, intact \(^{125}\)I-Tyr\(^{-}\)SRIF and ~25I-tyrosine were released at the same rate. Furthermore, the dissociation of both \(^{125}\)I-Tyr\(^{-}\)SRIF and ~25I-tyrosine followed simple first-order kinetics, and the same dissociation rate constant was observed whether equilibrium binding had been achieved or the dissociation reaction was initiated pre-equilibrium (32). These results indicate that the affinity state of the SRIF receptor does not change during the binding reaction and that both intact and degraded peptide dissociate from a single form of the receptor. The fact that intermediate degradation products were not found in the dissociation medium suggested that an aminopeptidase cleaved the NH\(_2\) terminal ~25I-tyrosine residue from \(^{125}\)I-Tyr\(^{-}\)SRIF. Such an activity has been reported in GH cells (12). Alternatively, intermediate degradation products may be too unstable to accumulate to detectable levels.

To characterize further the proteases that degrade receptor-bound \(^{125}\)I-Tyr\(^{-}\)SRIF, we examined the ability of different

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protease inhibitors to block the degradation. As stated previously, chloroquine and leupeptin did not inhibit [\(^{125}\)I-Tyr\(^*\)]SRIF degradation. Bacitracin, phenylmethylsulfonyl fluoride, TLCK, \(\alpha\)-macroglobulin, and puromycin had only a small effect. Only phosphoramidon, TPCk and the sulphydryl protease inhibitors iodoacetamide and chloromercurobenzoate produced substantial inhibition of [\(^{125}\)I-Tyr\(^*\)]SRIF degradation. Interestingly, sulphydryl protease inhibitors also reduced the nonlysosomal degradation of receptor-bound [\(^{125}\)I-Insulin in rat adipocytes (38). It must be kept in mind, however, that these inhibitors can have multiple targets and are quite toxic to cells. Therefore, the inhibitors may be eliciting their effects indirectly rather than by specific inactivation of degradative enzymes.

The importance of ligand-receptor internalization in the mechanism of action of peptide hormones and growth factors remains unresolved because an agent that selectively inhibits the process of receptor-mediated internalization without serious side effects has not been discovered. Since anti-receptor antibodies have been shown to mimic the effects of EGF (36) and insulin (18), internalization of these ligands was suggested to be unnecessary for their biological activity. However, since anti-receptor antibodies lead to the internalization of the receptors for both insulin (5) and EGF (37), receptor internalization may be required. We have demonstrated that [\(^{125}\)I-Tyr\(^*\)]SRIF is biologically active under conditions where it is not internalized (35). In addition, we have found that SRIF receptor number is increased rather than decreased by SRIF pretreatment (manuscript in preparation). The absence of internalization of either SRIF or its receptor under conditions where SRIF inhibits hormone secretion indicates that these processes are not required for SRIF action in GH\(_4\)C\(_{1}\) cells.

Reyl and Lewin have reported that most of the SRIF binding sites in rat pancreatic and gastric mucosal cells are cytosolic and have proposed that these binding sites may mediate the biological actions of SRIF by stimulating soluble phosphoprotein phosphatase activity (29, 30). We did not detect any high-affinity binding of [\(^{125}\)I-Tyr\(^*\)]SRIF to cytosolic proteins prepared from GH\(_4\)C\(_{1}\) cells. The absence of receptor-mediated uptake of [\(^{125}\)I-Tyr\(^*\)]SRIF to cytosolic proteins prepared from GH\(_4\)C\(_{1}\) cells suggests that, even if cytosolic receptors were present, they would not be accessible to SRIF. Therefore, these results further support the conclusion that the actions of SRIF are mediated directly by the plasma membrane receptor in GH\(_4\)C\(_{1}\) cells. In fact, the SRIF receptor present in membranes prepared from GH\(_4\)C\(_{1}\) cells inhibits adenylyl cyclase activity (19), and this effect clearly initiates at least some of the biological actions of SRIF (10, 33). However, the fact that the SRIF receptor is coupled to adenylyl cyclase does not explain its unique form of processing: other receptors that either stimulate (e.g., \(\beta\)-adrenergic receptors) or inhibit (e.g., enkephalin receptors) adenylyl cyclase have been shown to mediate rapid endocytosis of their respective ligands (2, 21, 39).

In summary, we have shown that the processing events that follow the initial binding of [\(^{125}\)I-Tyr\(^*\)]SRIF to its plasma membrane receptor differ markedly from those that follow binding of all other peptide ligands that have been studied in GH\(_4\)C\(_{1}\) cells. Receptor-bound [\(^{125}\)I-Tyr\(^*\)]SRIF remains at the cell surface while it elicits its biological effects and is partially degraded by membrane proteases, apparently simultaneously with dissociation. The importance of SRIF degradation in its mechanism of action and the consequences of the lack of rapid internalization for receptor regulation and desensitization present interesting questions for study.

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