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PARTIAL AMINO ACID SEQUENCE OF A SOMATOSTATIN RECEPTOR ISOLATED FROM GH_4C_1 PITUITARY CELLS

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SUMMARY: A somatostatin receptor isolated from GH_4C_1 rat pituitary tumor-derived cells was cleaved with cyanogen bromide or cyanogen bromide+trypsin to obtain sequenceable fragments. Five unique amino acid sequences ranging from 6 to 27 amino acid residues were obtained. The sequence was identical to sequence recently reported for one of two somatostatin receptors cloned from human pancreas [Yamada et al., (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 251-255] except for a single valine to isoleucine substitution. This is the first report of amino acid sequence from a purified somatostatin receptor.

Somatostatin (SRIF) peptides regulate a variety of different functions including hormone release in the pituitary (1) and pancreas (2), gut motility (3) and neural transmission (4). Evidence for SRIF receptor subtypes includes differential effects of the tetradecapeptide form, SRIF-14, the N-terminally extended form, SRIF-28 and other analogs in a variety of assays. This includes 3- to 100-fold differences in receptor binding affinity in brain (5,6) pancreas (2,7) and pituitary (8). Also there are differential effects on insulin and glucagon release in pancreas (9) and on K^+ channel activation in cultured cerebral cortical neurons (10).

Although purifications of SRIF receptors from brain (11) and intestinal tumor cells (12) have been reported, no amino acid sequence for these receptors has been published. However the recent molecular cloning and functional expression of two SRIF receptor subtypes from human pancreas by Yamada et al. (13) confirmed the existence of structurally distinct SRIF receptors with different tissue distributions. The structures reported by Yamada et al. contain

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ABBREVIATIONS: SDS-PAGE, sodium dodecylsulfate- polyacrylamide gel electrophoresis; PVDF, polyvinylidine difluoride; SRIF, somatostatin (somatotropin release inhibitory factor); hSSTR, human somatostatin receptor; CNBr, cyanogen bromide; PTH, phenylthiohydantoin.

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seven hydrophobic, transmembrane spanning sequences and are therefore consistent with the reported pharmacology of SRIF receptors, which indicates coupling to G proteins (14).

We have recently developed an effective method for purification of SRIF receptors via a N-terminally biotinylated SRIF-28 analog (C.M. Eppler et al., submitted for publication). Here, we present amino acid sequence from the purified GH_4C_1 cell SRIF receptor and compare it to almost identical sequence contained in hSSTR2, one of the human clones. This initial report of amino acid sequence from a pituitary SRIF receptor is especially significant since Yamada et al. (13) did not report a pituitary localization for either of their two clones.

EXPERIMENTAL PROCEDURES

Preparation of receptor. Pure SRIF receptor was prepared from about 2 grams of GH_4C_1 cell membrane protein. The purification method will be described in detail elsewhere (C.M. Eppler et al., submitted for publication). It consists essentially of the following steps: 1. preparation of a soluble, non-covalent complex between SRIF receptor from GH_4C_1 cell membranes and a biotinylated SRIF-28 analog; 2. purification of the receptor:ligand complex on streptavidin-agarose and 3. final purification of the receptor on wheat germ agglutinin agarose.

Cyanogen bromide cleavage. Purified receptor was separated from detergent and concentrated to a dry pellet in a 1.5 ml Eppendorf tube by the $CHCl_3$:MeOH extraction method of Wessel and Flugge (15). The protein pellet was dissolved in 100 µl of 70% formic acid (Aldrich) containing 1% cyanogen bromide (Aldrich) and the sample was incubated for 20h at room temperature. Solvents were then removed under vacuum in a Speed-Vac centrifuge (Savant) and the sample was either washed once with water and dissolved in SDS-sample buffer (16) or processed for typtic digestion as described below.

Trypsin digestion. For tryptic digestion, the CNBr digests were solubilized in 50 µl of 8M urea and then reduced and alkylated and trypsinized by the method of Stone et al. (17).

Analysis and purification of peptides. The CNBr fragments were separated on 16% pre-cast polyacrylamide gels (Novex, San Diego, CA) at 80 mA constant current in a running buffer containing 0.1% SDS, 0.1 M Tris and 0.1 M Tricine (pH 8.3). The gels were either stained with silver (18) on the analytical level or, on the preparative level, electroblotted by the method of Matsudaira (19) onto "ProBlott" PVDF membrane (Applied Biosystems, ABI). The peptide bands were then detected by Coomassie blue staining and excised from the membrane.

The CNBr/tryptic fragments were separated by reverse phase HPLC using a narrowbore Spheri-5 C-8 column (ABI, 2.1x100 mm) and a gradient of acetonitrile in 0.1% trifluoroacetic acid.

N-terminal sequence analysis. N-terminal sequence analyses by automated Edman degradation were carried out in an ABI model 477A protein sequencer. Sequencing of the CNBr fragments was performed directly on the peptide bands on PVDF in an ABI "Blott cartridge".

RESULTS AND DISCUSSION

Purified SRIF receptor appeared as a diffuse, 85 KDa band after SDS-PAGE and silver staining (Fig.1). The high purity of the 85 KDa glycoprotein is apparent. The molecular weight

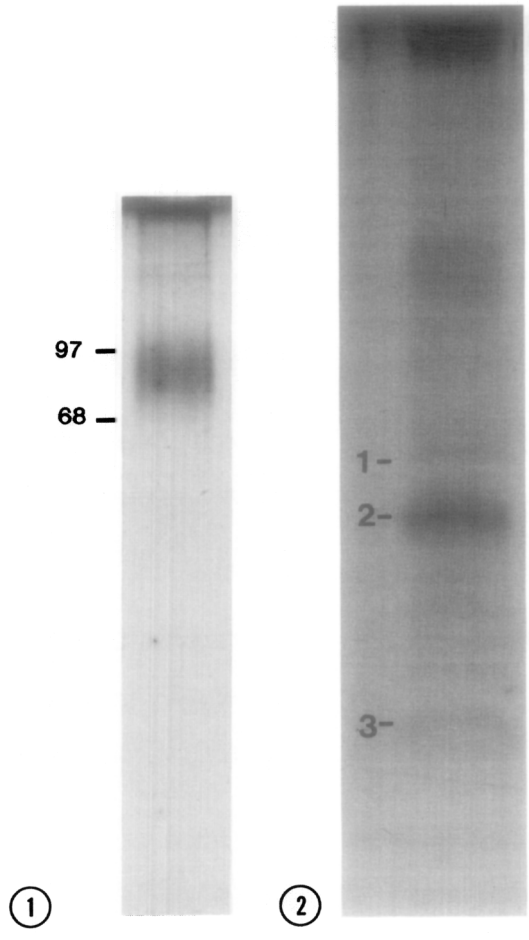

Figure 1. SDS-Polyacrylamide Gel Electrophoresis of Purified Somatostatin Receptor. Pure SRIF receptor was solubilized in SDS sample buffer, separated by SDS-PAGE on an 11% gel (16) and silver stained after prestaining with Alcian blue dye to enhance glycoprotein staining (20).

Figure 2. Electrophoretic Separation of Cyanogen Bromide Fragments of Somatostatin Receptor. SRIF receptor was digested with CNBr and the fragments were separated by SDS-PAGE on a 16% gel and silver stained as described in "Experimental Procedures". N-terminal amino acid sequence was obtained from peptides at positions labeled "1", "2" and "3" in the figure. By comparison with MW markers (prestained low MW set from BRL labs, Bethesda, MD) these positions correspond to MWs of about 10,000, 7,000 and 3,000 KDa.

is similar to that indicated by radiolabeling of GH_4C_1 cell membrane proteins with a photoactivatable [^{125}I]-labeled SRIF analog (21).

After CNBr digestion, several fragments in the MW range from 3-10 KDa were resolved by SDS-PAGE on 16% tricine gels (Fig. 2; silver-stained analytical gel). The peptides at positions 1, 2 and 3 in Fig. 2 (MWs of about 10, 7 and 3 KDa) were the receptor fragments that yielded clear N-terminal sequence. Band 1 was not stained well with silver at the analytical level but was visibly stained by Coomassie blue on the PVDF blot of the preparative gel (not shown). CNBr

Table 1. Amino acid sequences of cyanogen bromide and cyanogen bromide/tryptic fragments obtained from the GH_4C_1 cell somatostatin receptor. Peptide fragments from the purified SRIF receptor were generated, purified and analyzed by N-terminal sequencing as described in "Experimental Procedures". Five of the sequences obtained are shown here and compared with selected sequences from the human SRIF receptor clones, hSSTR1 and hSSTR2, recently described by Yamada et al. (13). In the GH_4C_1 sequence, lower case letters denote some ambiguity in the call and "X" no call. "·" indicates identity to the hSSTR2 sequence.

A. GH_4C_1/CNBr1	iYAgLRXNQXG
	····· ·· ·
hSSTR2 (179-189)	IYAGLRSNQWG
	· ·
hSSTR1 (194-204)	VFSRTAANSDG
B. GH_4C_1/CNBr2	FDFVVILTYANsXANPILYAFlXDNfk
	····· ····· ····· ····
hSSTR2 (293-320)	FDFVVVLTYANSCANPILYAFLSDNFK
	· · ····· ·····
hSSTR1 (305-332)	SQLSVILGYANSCANPILYGFLSDNFK
C. GH_4C_1/CNBr3	sIDRYLAVVHPIKSAkXRr
	····· ··
hSSTR2 (137-155)	SIDRYLAVVHPIKSAKWRR
	· ··· ····· · ··
hSSTR1 (152-170)	SVDRYVAVVHPIKAARYRR
D. GH_4C_1/CNBr/Tryp.1	LNETTETQR
	·····
hSSTR2 (350-358)	LNETTETQR
	·
hSSTR1 (362-370)	AYSVEDFQP
E. GH_4C_1/CNBr/Tryp.2	TITNIY
	·····
hSSTR2 (76-81)	TITNIY
	· ····
hSSTR1 (91-96)	TATNIY

digestion followed by tryptic digestion generated additional peptides that were resolved by reverse-phase HPLC (not shown).

Table 1 shows the N-terminal amino acid sequences obtained from the fragments. These sequences are compared to sequences present in hSSTR1 and hSSTR2, the two human SRIF receptor clones of Yamada et al. (13). From 72 sequencing cycles carried out on five different peptides, there were 58 unambiguous calls, 9 calls with some degree of ambiguity and 5 cycles with no call. All of the cycles with no call correspond to serine, cysteine or tryptophan in the hSSTR2 sequence. These amino acids are difficult to detect at the low picomole level, especially

from PVDF membrane. The amino acid calls were based on amounts of PTH-amino acids ranging from 6-38 picomoles. The CNBr1 sequence was the lesser of two sequences from that band on a quantitative basis. However the major sequence was the same as in CNBr2, which yielded a single clear sequence, and could therefore be easily subtracted out of the data from CNBr1.

Two immediately apparent, general homologies with the G protein coupled receptors were the sequences DRY and NPILY (in CNBr3 and CNBr2 respectively). The DRY sequence occurs in intracellular loop II of most G protein coupled receptors while NPXXY, with X indicating a hydrophobic amino acid residue, is a characteristic of transmembrane region VII (22). The near identity between our CNBr sequences and regions of the hSSTR2 sequence found by Yamada et al. (13) provide even more convincing evidence that we have purified a SRIF receptor subtype. The only difference found between the GH_4C_1 sequence and hSSTR2 was a substitution of isoleucine for valine at position 298 of hSSTR2. CNBr2, CNBr3 and CNBr/Tryp.2 also had significant homology with hSSTR1, 72%, 78% and 85% respectively. It is probable therefore that the SRIF receptor from GH_4C_1 cells is the rat homologue of the SSTR2 subtype.

Although the sequence obtained from the GH_4C_1 cell SRIF receptor is of obvious utility in molecular cloning, the overall homologies between this rat pituitary receptor, hSSTR1 and hSSTR2 remain to be seen. Since this is only the first report of amino acid sequence from a pituitary SRIF receptor, the existence of multiple subtypes within the pituitary is also a possibility.

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