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Covalent peptide transfer to cell membrane proteins (peptidyl transferase)

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HL60 cells, rabbit peritoneal granulocytes or membrane preparations of these cells incorporate radioactivity when reacted with the radioactive peptide tuftsin [^{3}H Pro^3]-Thr-Lys-Pro-Arg. The radioactivity which is not diminished by repeated treatments with TCA and NaOH, is covalently bound to a membrane acceptor protein of 100 kDa. The peptide is not displaced by large concentrations of its constituent amino acids. The acceptor protein is resolved into one labeled peak by gel filtration on Sephadex G-200, Sephacryl S-300 and by SDS-PAGE. Digestion by trypsin and chymotrypsin results in the production of smaller fragments.

Tuftsin; Oligopeptide; Membrane protein; Covalent bond

1. INTRODUCTION

The incubation of the tetrapeptide [^{3}H Pro^3]-Thr-Lys-Pro-Arg, tuftsin [1] with washed HL60 cells, rabbit peritoneal granulocytes or membranes prepared from these cells results in binding of radioactivity to membrane proteins. This binding is of two types. One type which has been studied extensively, is dissociable and exhibits specific membrane receptors [2–7]. The other type of binding is nondissociable. It is the latter that is the subject of this communication.

2. MATERIALS AND METHODS

Bio-Gel, Bio-Rad Laboratories, Rockville Centre, NY; Gen-Probe, San Diego, CA; Sephadex and Sephacryl, Pharmacia, Piscataway, NJ; HL60, a human cell line, ATCC, Rockville, MD; RPMI 1640 culture medium, Sigma Chemical Co., St. Louis, MO; FCS, Hyclone Laboratory, Logan, UT; [^{3}H Pro^3]-Thr-Lys-Pro-Arg, New England Nuclear, Boston, MA; nonradioactive tuftsin was synthesized in our laboratory [6]; glutamine, penicillin and streptomycin, Irvine Scientific, Santa Ana, CA; HL60 cells were grown in RPMI 1640 with 10% FCS, 2 mM glutamine, 100 U/μl penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C; HL-60 membranes were prepared as before [6]. All manipulations were carried out in the presence of protein inhibitors, phenylmethylsulfonyl fluoride (2 mM), aprotinin (0.5 trypsin inhibitor unit/ml), pepstatin (2.5 μg/ml), antipain (1 μg/ml), leupeptin (20 μM) and 0.02% sodium azide.

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Abbreviations: ATCC, American type culture collection; FCS, fetal calf serum; DTT, dithiothreitol; HL60, human leukemia cell line; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; TCA, trichloroacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid

3. RESULTS AND DISCUSSION

It appears that the nondissociable radioactivity is due to covalent bond formation between the tetrapeptide or a portion thereof and a single acceptor membrane protein that acquires radioactivity, during the reaction. This incorporated radioactivity displays typical features indicative of a covalent bond formation. The radioactive acceptor protein can be repeatedly precipitated with TCA and dissolved in NaOH without loss of radioactivity.

The incorporated radioactivity resists displacement by alanine or proline singly at a concentration of a thousandfold that of the added

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Table 1

The incorporation of radioactivity from [^{3}H Pro^3]-Thr-Lys-Pro-Arg into HL60 and rabbit peritoneal granulocyte membrane protein or its equivalent of washed cells

Reaction condition	cpm	Δcpm	fmol[a] incorporated
Heat killed	288	0	0
0°C	299	0	0
22°C	836	548	27.4
37°C	808	520	26.0
HL60 cells 2×10^7	748	460	23.0
Alanine added (25 μM)	876	589	29.4
Proline added (25 μM)	868	580	29.0
Amino acids T, K, P, R (25 μM)	880	592	29.6
Thr-Lys-Pro-Arg (25 μM)	388	100	5.0
Thr-Lys-Pro-Arg (25 μM)	396	108	5.4
Rabbit membrane granulocyte	768	480	24.0
Rabbit membrane granulocyte	596	418	20.97
Rabbit membrane granulocyte	496	208	10.4

[a] fmol = 20 cpm

The transfer of radioactivity from [^{3}H Pro^3]-Thr-Lys-Pro-Arg to an acceptor membrane protein from HL60 and rabbit peritoneal granulocytes. The reaction mixture (1 ml) consisted of 1 mg of membrane protein, 1×10^5 cpm (5 pmol), 0.25 M:10^{-3} M sucrose/phosphate, pH 7.4, 22°C, reaction 1 h. This was terminated by 10% cold TCA. The precipitate was alternatively dissolved in 0.2 N NaOH followed by TCA precipitation. This cycle can be repeated 4 more times without loss of radioactivity. Controls were made by incubation at 0°C or by incubation with heat denatured membrane preparations. Incorporation was not affected by high concentrations of alanine or proline. Absence of any effect is shown with threonine, lysine, proline and arginine added together also in high concentration. In contrast, the incorporation is shown to be considerably reduced by adding the intact nonradioactive tetrapeptide. HL60 cultures were regularly tested for mycoplasma using [^{3}H]DNA homologous to mycoplasma and acholeplasma ribosomal RNA supplied by 'Gen-probe'. All rabbit granulocyte membranes used were kept frozen for 6 or more months

radioactive peptide, and by the combined constituent amino acids, threonine, lysine, proline and arginine at the same high concentration. In contrast, the radioactive incorporation is greatly inhibited by the intact nonradioactive tetrapeptide (table 1). Furthermore, electrophoresis on SDS-PAGE and gel filtration on Sephacryl S-300 or Sephadex G-200 yielded only one radioactive component of about 100 kDa (fig.1). Proof that the radioactive component is indeed a protein is shown by the fact that digestion with trypsin or

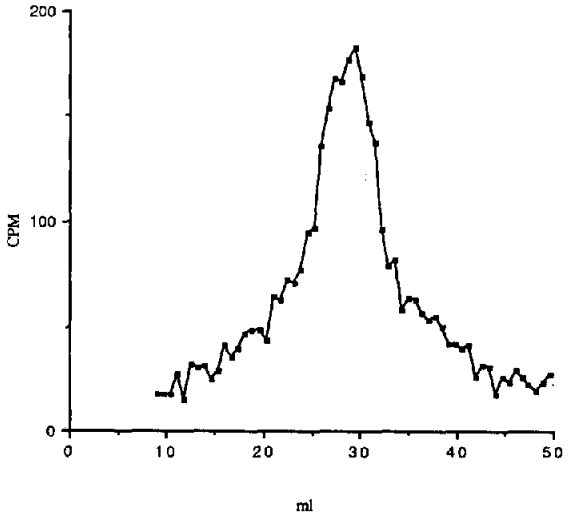

Fig.1. Approx. 20 mg of HL60 membrane protein were reacted with [^{3}H Pro^3]-Thr-Lys-Pro-Arg, 1×10^6 cpm (50 pmol), in 5 ml of 0.25 M:5×10^{-3} M sucrose/phosphate, pH 7.4, at 22°C for 1 h. The reaction was stopped by the addition of ice-cold 10% TCA, left on ice for 15 min and processed as in table 1. The pH was adjusted to 8.8, with a final volume of 2 ml. It was reduced with 50 mM DTT, alkylated with 5 mM iodoacetamide, each for 1 h at 37°C. Tris was added to 0.1 M, pH 8.8, and sample boiled in 1% SDS. The column, Sephacryl S-300, 22 × 1.5 cm, was equilibrated in 0.1 M Tris, pH 8.8, and 1% SDS, loaded with approx. 1500 cpm of the solubilized sample. Filtration rate was 15 ml/h, 0.7 ml fractions were counted. Column was calibrated with (kDa) 667 thyroglobulin, 158 γ-globulin, 43 ovalbumin, 17 myoglobin and 1.3 cyanocobalamin. The mobility of the radioactive peak corresponded to approx. 100 kDa.

chymotrypsin yielded smaller radioactive fragments (figs 2 and 3).

In view of the results shown in table 1 and the molecular mass of the radioactivity shown in figs 1–3, we may conclude that the tetrapeptide [^{3}H Pro^3]-Thr-Lys-Pro-Arg, under the conditions employed, is incorporated whole or in part through a covalent linkage to an acceptor protein of about 100 kDa. The mechanism of the covalent insertion remains to be determined. The reaction required no added energy source such as ATP. However, the reaction may well proceed, with little change in ΔF, through a peptide bond exchange between the tetrapeptide and the acceptor protein similar to that obtained in murein synthesis [8].

Peptide bond exchange may take place at the amino- or carboxy-terminal of the oligopeptide or the membrane protein. In this case, it would re-

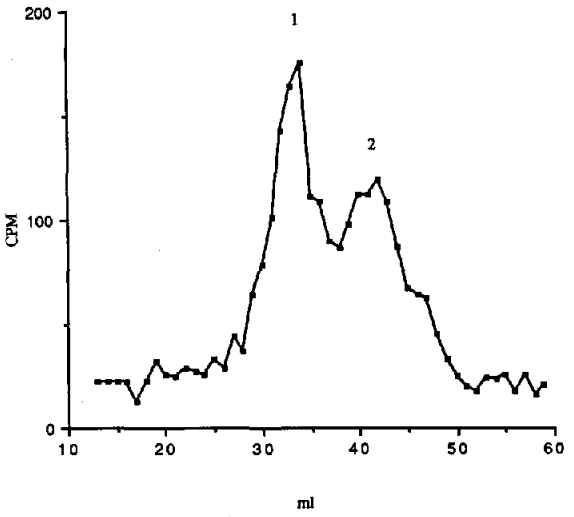

Fig.2. Membranes (20 mg) were prepared from HL60 cells, reacted with [^{3}H]tuftsin in 5 ml of sucrose phosphate buffer, pH 7.4, and treated with TCA, NaOH, reduced and alkylated, all as in fig.1. pH adjusted to 8.5 in 0.1 M ammonium carbonate and treated with 1.0 mg of trypsin in 1 mM $CaCl_2$ for 2 h at 37°C and then boiled in 1% SDS. Approx. 2000 cpm were loaded on a Sephacryl column S-300, 25 × 1.5 cm equilibrated in 1% SDS and 0.1 M ammonium carbonate. 1.0 ml of effluent was counted. Molecular mass standards as in fig.1. Peak 1 and peak 2 represent 100 and 18 kDa, respectively.

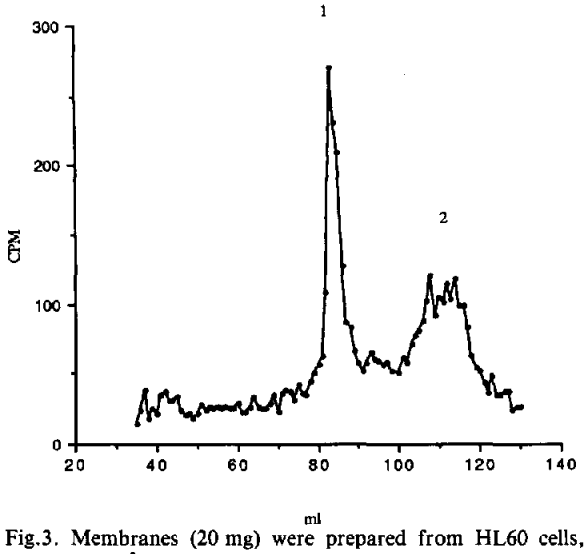

Fig.3. Membranes (20 mg) were prepared from HL60 cells, reacted with [^{3}H]tuftsin and treated with TCA, NaOH, reduced and alkylated as in fig.1. pH adjusted to 8.5 in 0.1 M ammonium carbonate and treated with 1.0 mg of chymotrypsin for 24 h at 37°C and then boiled in 1% SDS. Approx. 2000 cpm were loaded on a Bio-Gel A 1.5 m column, 55 × 1.5 cm, equilibrated in 1% SDS and 0.1 M ammonium carbonate. 1.0 ml of effluent was counted. Molecular mass standards as in fig.1. Peak 1 and peak 2 represent 18 kDa and less than 1 kDa, respectively.

quire the elimination of one or more residues from the particular terminal, and simultaneously shortening or lengthening one chain or the other as the case may be. However, if the exchange reaction takes place within the body of the protein chain then that particular chain would break at the point of exchange signalling a novel pathway for fragmenting protein by small peptides through this reaction.

It is assumed here that the reaction we are studying is catalyzed by a specific enzyme, as the reaction is highly influenced by the pH and the buffer used. No activity can be elicited at 0°C but the process is active at room temperature and at 37°C. Optimum activity is obtained at about pH 8.5 in 0.1 M Hepes buffer. No metal requirement could be elicited. To our knowledge this reaction has not been described in mammalian tissue.

It must be stated at the outset that the reaction described herein does not necessarily imply any specificity for the tetrapeptide tuftsin. Indeed it is likely to be a general phenomenon that would define yet another posttranslational modification of a protein for possible modulation of its biological activity. This would be similar to phosphorylation, adenylation and methylation.

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