Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 535-541

PROCESSING OF THE HUMAN IM-9 LYMPHOBLAST SUBSTANCE P RECEPTOR

Biosynthetic and Degradation Studies Using a Monoclonal Anti-Receptor Antibody

Michele L. Organist, Jeanne P. Harvey,
Joseph P. McGillis, and Donald G. Payan

Howard Hughes Medical Institute and Departments of Medicine (Division of Allergy and Immunology) and Microbiology, University of California Medical Center, San Francisco, California 94143-0724

Received January 11, 1988

Cellular membrane receptors for the immunostimulatory neuropeptide substance P have been previously identified on the cultured lymphoblast cell line, IM-9. The regulation of this receptor by ligand and the contribution to its molecular weight by N-linked sugars was studied by incubating IM-9 cells for 14 hr in the presence of [^{35}S]met with or without substance P and tunicamycin, respectively. Cells were lysed and the receptor proteins were immunoprecipitated with an anti-receptor monoclonal antibody. SDS-PAGE analysis of untreated cellular lysates revealed specifically precipitated proteins of 38 kD and 33 kD, which were downregulated by substance P. In tunicamycin-treated cells, whose substance P binding was not affected, the major immunoprecipitated protein had an apparent M_r of 29 kD. The time course of receptor processing was studied by pulse chase analysis. Three proteins of molecular weights 38 kD (mature receptor), 36 kD and 33 kD (receptor precursors) were identified for time periods of 30 min to 4 hr. The half life of the mature receptor and its precursors was approximately 1 hr and 0.5 hr, respectively. Results from the present studies suggest that the lymphocyte substance P receptor is translated as a precursor protein that is glycosylated.

Substance P (SP) exhibits important physiological activities in the nervous, endocrine, and immune systems (1). The immunomodulatory properties of SP include the stimulation of proliferation of both human (HPBTL) (2) and murine (3) T-lymphocytes in the presence and absence of mitogens by nanomolar concentrations of SP. Similar stimulatory effects on the uptake of [^{3}H] thymidine was observed when SP was incubated with murine lymphocytes from

Abbreviations used: SP, substance P; HPBTL, human peripheral blood T-lymphocytes; PP, Peyer's patch; SP*, the fluorescent conjugate of SP; FACS, fluorescence-activated cell sorter; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecylsulfatepolyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; NP40, Nonidet P-40; and SDS, sodium dodecylsulfate.

0006-291X/88 $1.50

spleen, mesenteric lymph nodes, and Peyer's patches (PP). In addition, SP significantly increased the *in vitro* production of IgA in lymphocytes from spleen and PP by as much as 300% (3). More recently it has been shown that T-lymphocytes from the synovial fluid of patients with rheumatoid arthritis showed a five-fold mitogen-induced proliferative response when exposed to SP (4). Biochemical characterization of the actions of SP at the level of lymphocyte membranes has shown that SP stimulated the incorporation of [^{32}P] into membrane phospholipids by activating the phosphatidylinositol pathway (5).

Specific functional receptors for SP have been demonstrated on HPBTL and murine lymphocyte subsets using [^{3}H]SP, [^{125}I]SP, and a fluorescent conjugate of SP (SP*) (6). Two-color fluorescence-activated cell sorter (FACS) analysis demonstrates that approximately 20-30% of HPBTL recognize SP*. The K_D for [^{3}H]SP binding to these cells was 1-2 nM, with approximately 20,000 receptors/SP* cell. Similar affinities and cell distribution of SP-reactive cells was demonstrated in murine lymphocytes, where Peyer's patch lymphocytes have a greater number of SP* cells than splenocytes (7). The cultured lymphoblast line IM-9 demonstrates the presence of a single class of approximately 20,000 SP receptors/cell with a K_D of 0.6 nM (8). The IM-9 lymphoblast SP-receptor has been more fully characterized biochemically by the demonstration of the solubilization of SP binding activity from IM-9 cell membranes (9). Affinity crosslinking studies demonstrate that [^{125}]SP specifically binds to membrane proteins of apparent molecular weight 33 and 58 kD. Peptide mapping of these membrane protein constituents reveals that the principal binding domain is located on the 33 kD protein. This membrane protein has been purified to apparent homogeneity by indirect immunoaffinity chromatography (10) and a monoclonal antibody against this SP-binding membrane constituent has been developed (11). In this study, the biosynthesis and degradation of the IM-9 lymphocyte SP receptor is studied using a monoclonal antibody against an epitope on the 33 kD SP-binding protein.

MATERIALS AND METHODS

Materials

RPMI 1640, penicillin (1000 U/ml), streptomycin (1000 μg/ml), and methionine-free RPMI 1640 (UCSF Cell Culture Facility); L-methionine and fetal bovine serum (Gibco); [^{35}S]methionine (>1000 Ci/mmole) and Amplify (Amersham); tunicamycin and Pansorbin Staph A cells (10% w:v) (Cal Biochem); phenylmethylsulfonyl fluoride (PMSF), 1,10 phenanthroline, and ethylenediaminetetraacetic acid (EDTA) (Sigma); substance P (SP) and chymostatin (Peninsula Labs); SDS-PAGE reagents (BioRad); Kodak 70 XO mat AR film (Eastman Kodak); Dupont Cronex lightning-plus enhancing screens (E.I. DuPont de Nemours and Co.); and cultures of human IM-9 lymphoblasts (American Type Culture Collection) were obtained from the designated suppliers.

Methods

Cell Culture and Metabolic Labeling of SP Receptors

IM-9 lymphoblast cells were grown in 850 cm^2 tissue culture roller bottles at a density of 2.0 x 10^6 cells/ml in RPMI 1640 supplemented with

L-glutamine, 10% (v:v) heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium) at 37°C in 5% CO_2: 95% air. The cultures were divided every 48-72 hr and viability always exceeded 95% as determined by the exclusion of trypan blue dye.

For metabolic labeling, cells that reached a stationary phase of growth were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS). Cells were resuspended in fresh methionine-free or glucose-free RPMI medium at a density of 10^7 cells/ml and incubated 30 min at 37°C before the addition of [^{35}S]methionine (0.1 mCi/ml). Incubation at 37°C was continued for 4-5 hr. In some cases, cells were pretreated with 3 μg/ml of tunicamycin for a 6 hr period prior to metabolic labeling. During labeling tunicamycin at 2 μg/ml was present at all times.

Down-Regulation and Pulse Chase Analysis

Stationary growth phase IM-9 cells were washed with PBS and resuspended at 2 x 10^6 cells/ml in fresh complete RPMI 1640. Cells were preincubated for 12 hr at 37°C in medium without and with 10^{-6}M SP. Following preincubation, cells were washed twice with PBS and resuspended at 1 x 10^7 cells/ml in methionine-free RPMI supplemented with 1 x 10^{-6}M SP. Following incubation for 30 min at 37°C, the cells were resuspended in fresh medium with [^{35}S]methionine (0.1 mCi/ml). The cells were then pulsed for 20 min, washed twice with PBS and resuspended at 2 x 10^6 cells/ml in complete RPMI supplemented with 14 mM methionine. Cells were washed three times in PBS and lysed with 10 mM Hepes (pH 7.2), 0.2 M NaCl, 2.5 mM $MgCl_2$, 1% Triton, 0.1 mM PMSF, and 0.1 mM leupeptin. Lysates were cleared by centrifugation at 400,000 x g for 16 min and the supernatants used for immunoprecipitation studies.

Immunoprecipitation of SP Receptors and SDS-PAGE Analysis

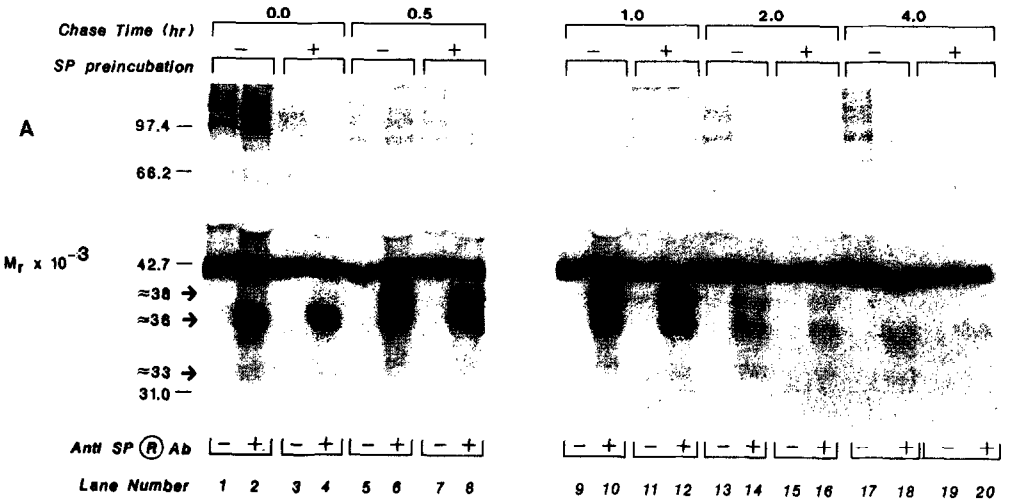
IM-9 cell lysates (0.1 ml) were incubated with either anti-substance P receptor IgG (anti-SPR) or control mouse IgG at 10 μg/ml for 12-16 hr at 4°C. Fifty μl of a 10% solution of Staph A cells were added and incubation continued for 1 hr. Cells were pelleted by centrifugation at 3500 x g for 20 min at 4°C. Pellets were washed five times with 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.1 M NaCl, 5 mM EDTA, 0.02% Na azide, 1 mM PMSF, and 50 mM Tris-HCl (pH 7.4). Immune complexes were dissociated by boiling for 5 min in sample buffer [0.125 M Tris-HCl (pH 6.8), 4% SDS, 0.005% bromophenol blue, 5% dithiothreitol, and 30% glycerol].

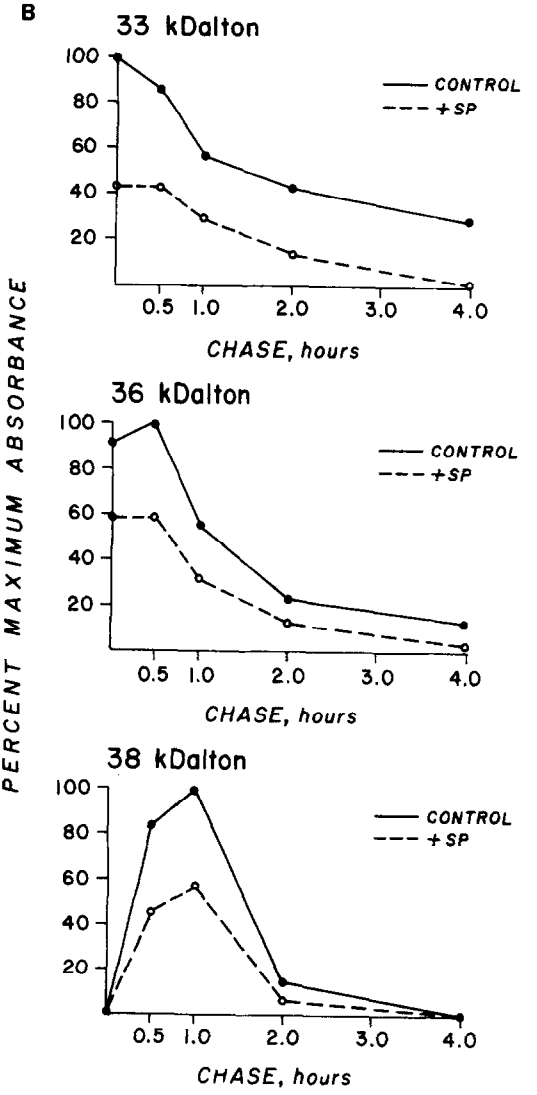
Electrophoretic separation was carried out according to the methods of Laemmli (12) with separating gels of 7.5% and 10%, and stacking gels of 5%. Gels were stained in 0.025% Coomassie Brilliant Blue R in 50% methanol: 5% acetic acid (v:v). Destaining took place overnight in 7.5% acetic acid. For the [^{35}S] labeled proteins, gels were incubated in Amplify for 30 min and then dried onto filter paper. Autoradiography was conducted at -70°C with Kodak XAR-5 film and a DuPont Cronex intensifying screen for 3-10 days. Scanning densitometry was performed using a Zeineh Soft Laser Scanning densitometer.

RESULTS AND DISCUSSION

Pulse Chase Analysis and Down-Regulation of the Substance P Receptor

The biosynthesis and time-course of processing of the SP receptor was determined by pulse chase analysis in IM-9 cells. Cell lysates were analyzed with an anti-SP receptor antibody using immunoprecipitation. In addition, in order to investigate the effect of SP on specifically-precipitated receptor proteins, IM-9 cells were downregulated with 10^{-6}M SP for 12 hr prior to metabolic labeling. Fig. 1A is an autoradiogram of an SDS-PAGE analysis from the immuno-


Chase Time (hr)
0.0
0.5
1.0
2.0
4.0
SP preincubation
A
97.4
66.2
Mr x 10-3
42.7
≈38
≈36
≈33
31.0
Anti SP (R) Ab
Lane Number
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20


B
33 kDalton
CONTROL
+SP
PERCENT MAXIMUM ABSORBANCE
CHASE, hours
36 kDalton
38 kDalton

precipitates. Cells that were either not treated or downregulated with SP were pulsed for 20 min with [^{35}S]methionine. For immunoprecipitation, either control mouse IgG (-) or anti-SPR (+) was added to each time point cell lysate. At time 0, specifically-precipitated proteins of 33 kD and 36 kD are seen. Upon chasing for 30 min, a completely processed 38 kD form of the receptor appears. At 60 min of chase, over 50% of the radioactivity has been converted to the 38 kD protein. Densitometric analysis of this autoradiogram is seen in Fig. 1B. Incorporation of [^{35}S]methionine reaches a peak for the 36 kD receptor protein after 30 min and for the mature 38 kD receptor protein after 60 min. The 33 kD protein achieves its maximum at time 0 and declines from that point onward.

Preincubation with SP reduced the amount of [^{35}S]methionine incorporated by over 50% in the 33 kD protein and by 40% in the 36 kD and 38 kD proteins (Fig. 1, lanes 4, 8, 12, 16, 20). In addition, preincubation of IM-9 cells with anti-SPR Fab fragments also downregulated the amount of [^{35}S] methionine incorporated into the above SP receptor proteins (data not shown).

Tunicamycin Treatment of the Substance P Receptor

In order to examine for the presence of N-linked sugars on the lymphocyte SP receptor, IM-9 cells were cultured in the presence or absence of 3 μg/ml of tunicamycin. Tunicamycin has been shown to specifically inhibit the synthesis of N-acetylglucosaminyl pyrophosphoryl polyisoprenol leading to the inhibition of protein glycosylation (13). Fig. 2 demonstrates a pulse chase experiment carried out in a manner analogous to that in Fig. 1. Cells were pulsed for 20 min with [^{35}S]methionine, washed, and then chased for 0, 0.5, and 1.5 hr. Cellular proteins were immunoprecipitated with the monoclonal anti-SP receptor antibody and analyzed by SDS-PAGE and autoradiography. Following tunicamycin treatment, the 36,000 M_r precursor receptor protein had an apparent M_r of 29,000 (lane 4). However, following tunicamycin treatment, two faint but distinct bands could still be seen at 36,000 and 33,000,

---

Fig. 1. Pulse chase analysis and downregulation of the substance P receptor. IM-9 cells which had been preincubated for 12-14 hr in the presence or absence of 10^{-6}M SP were washed and pulsed for 20 min with [^{35}S]methionine in the presence or absence of 10^{-6}M SP. Cells were then washed and incubated (chased) for an additional 0, 0.5, 1, 2, or 4 hr in the presence of cold methionine. Immunoprecipitated membrane proteins were then analyzed by SDS-PAGE and autoradiography. (A) Pulse chase analysis of metabolically labeled SP receptor protein shows that an immature form of M_r 33,000 is processed up to 36,000 and 38,000 M_r forms. Preincubation with 10^{-6}M SP results in the significant downregulation of the SP proteins. (B) Densitometric analysis of the autoradiogram in 1A shows that the 36,000 M_r precursor peaks at 30 min, and the 38,000 M_r form at 1 hr. The half-life of the mature lymphocyte SP receptor is approximately 1 hr.

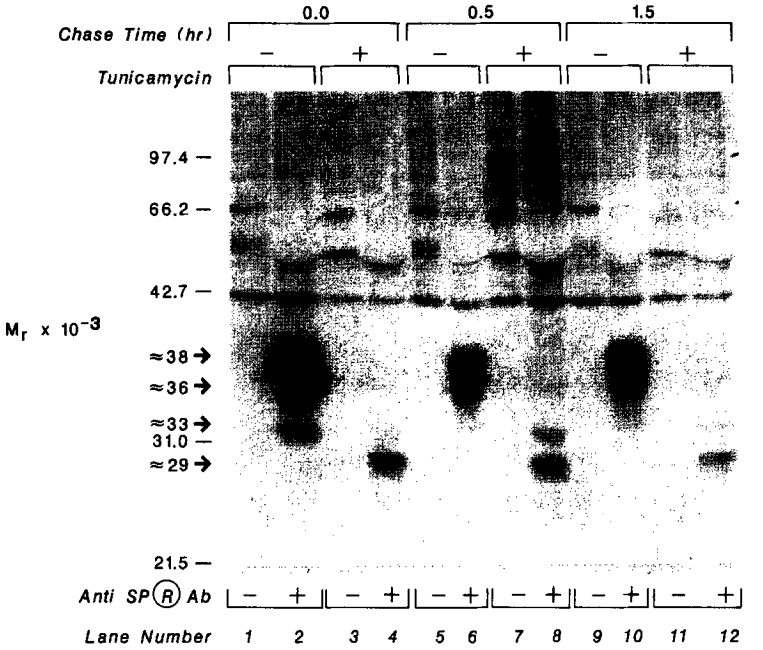


Fig. 2. **Effect of tunicamycin treatment on the substance P receptor.** IM-9 cells were cultured in the presence or absence of tunicamycin (3 μg/ml) for 6 hr prior to metabolic labeling. Following a 20 min pulse with [^{35}S]methionine, the cells were washed and chased for 0, 0.5, or 1.5 hr. Following tunicamycin treatment, the major immunoprecipitated protein had an apparent M_r of 29,000 (lanes 4, 8 and 12), whereas in the absence of tunicamycin, the predominant bands were the 36,000 and 38,000 M_r proteins (lanes 2, 6, and 10).

suggesting the presence of complex sugars on the receptor that are not N-linked. After 0.5 hr of chase, treatment of the 38,000 M_r mature form of the receptor with tunicamycin also results in a protein of 29 K_D with a stronger band at 33 K_D (Fig. 2, lane 8). However, by 15 hr of chase, tunicamycin treatment of the mature 38 K_D receptor results in the almost complete disappearance of the 33 Kd intermediary proteins (Fig. 2, lane 12). These results suggest that the SP receptor intermediates may exhibit non-N-linked sugars. When the binding of [^{125}I]SP to tunicamycin treated and untreated IM-9 cells was examined, no significant differences were detected in the levels of total and nonspecific binding (data not shown).

The experiments described here provide evidence that the lymphocyte SP receptor is synthesized as a distinct precursor, similar to other membrane associated receptor proteins for ligands such as insulin (14) and platelet-derived growth factor (15). The precursor is synthesized rapidly and is downregulated by SP. Moreover, treatment with the glycosylation inhibitor tunicamycin demonstrates that the mature form of the SP receptor exhibits significant degrees of glycosylation. These results will help to further elucidate the role of this receptor in modulating immune responses.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health grant NS-21710.

REFERENCES

1. Payan, D.G., McGillis, J.P., and Goetzl, E.J. (1986) Advances in Immunology, 39, Dixon, F.J., Austen, K.F., Hood, L., and Uhr, J.W., edss, Academic Press, New York, 299-323.

2. Payan, D.G., Brewster, D.R., and Goetzl, E.J. (1983) J. Immunol. 131, 1613-1615.

3. Stanisz, A.M., Befus, D., and Bienenstock, J. (1986) J. Immunol. 136, 152-156.

4. Lotz, M., Carson, D.A., and Vaughn, J.H. (1987) Science 235, 893-895.

5. Payan, D.G., and Goetzl, E.J. (1987) Am. Rev. Respir. Dis. (Proceedings of Meeting held 1/15/87 in Boca Raton, FL) , in press.

6. Payan, D.G., Brewster, D.R., Missirian-Bastien, A., and Goetzl, E.J. (1984) J. Clin. Invest. 74, 1532-1539.

7. Stanisz, A.M., Scicchitano, R., Dazin, P., Bienenstock, J., and Payan, D. G. (1987) J. Immunol. 139, 749-754.

8. Payan, D.G., Brewster, D.R., and Goetzl, E.J. (1984) J. Immunol. 133, 3260-3265.

9. Payan, D.G., McGillis, J.P., and Organist, M.L. (1986) J. Biol. Chem. 261, 14321-14329.

10. McGillis, J.P., Organist, M.L., and Payan, D.G. (1987) Anal. Biochem. 164, 502-513.

11. Organist, M.L., Harvey, J.P., McGillis, J.P., Mitsuhashi, M., Melera, P., and Payan, D.G. (1987) J. Immunol. 139, 3050-3054.

12. Laemmli, U.K. (1970) Nature 227, 680-685.

13. Takasuki, A., Kohno, K., and Tamura, G. (1975) Agri. Biol. Chem. 39, 2089.

14. Hedo, J.A., Kahn, C.R., Hayashi, M., Yamada, K.M., and Kasuga, M. (1983) J. Biol. Chem. 258, 10020-10026.

15. Keating, M.T., and Williams, L.T. (1987) J. Biol. Chem. 262, 7932-7937.