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FUNCTIONAL ACTIVITY OF A BIOTINYLATED HUMAN NEUROKININ 1 RECEPTOR FUSION EXPRESSED IN THE SEMLIKI FOREST VIRUS SYSTEM

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The 1.3 S biotinylatable subunit of *Proprionibacterium shermanii* transcarboxylase complex was fused to the C-terminus of the human neurokinin 1 receptor gene and introduced into the Semliki Forest virus expression vector pSFV1. RNA transcribed from pSFV1-NK1-biot and pSFV-Helper2 was co-electroporated into BHK cells permitting *in vivo* packaging of recombinant virus. Infection of BHK and CHO cells with SFV-NK1-biot virus yielded high level of the fusion receptor as detected by metabolic labeling, immunoblotting with streptavidin alkaline phosphatase and binding to substance P. Like native receptor, the biotinylated receptor fusion was able to stimulate Ca^{2+} mobilization in infected CHO cells,indicating functional coupling to guanine-nucleotide-binding proteins.
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The substance P or neurokinin 1 receptor belongs to the superfamily of guanine-nucleotide-binding protein (G protein)-coupled receptors with a postulated seven-transmembrane domain topology (1, 2). Neurokinin 1 has been identified in both the peripheral and central nervous system and is thought to play an important role in pain transmission and neurogenic inflammatory diseases (3, 4). The membrane topology, ligand binding and interaction with G proteins (5-7) have been extensively studied.

Seven-transmembrane receptors have been expressed in a variety of systems, including *Xenopus laevis* oocytes (8), bacteria (9), yeast (10) as well as insect (11) and mammalian cells (12, 13). An obstacle in generating large amounts of recombinant receptors has been the downstream processing to solubilize and purify these receptors from the plasma membrane. Several systems were developed by designing fusions with various tags that allow one-step purification using resins with affinity for the tag (14, 15). In such approaches, the choice of a

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resin and tag combination that permits the release of the fusion protein under mild conditions to preserve the activity of the recombinant protein is of great importance.

In this paper we use the Semliki Forest virus system (16, 17) to compare the expression and functional properties of the human neurokinin 1 receptor (NK1) with that of a fusion derivative, NK-1-biot, which carries the biotinylated peptide of the *Proprionibacterium shermanii* transcarboxylase complex (18) at the C-terminus. Our results show that the two forms are equally well expressed in BHK and CHO cells. Moreover, the two forms exhibit undistinguishable patterns of binding to substance P as well as functional (Fura-2) properties, thus validating the use of a biotinylated tag in the study of seven-transmembrane receptors.

MATERIALS AND METHODS

Materials. Plasmids were propagated in *Escherichia coli* DH5α and purified by standard procedures (19). Baby hamster kidney (BHK) and Chinese hamster ovary (CHO) cells from the ATCC collection were grown routinely in D-MEM-Iscove medium (Gibco-BRL) containing 10% fetal calf serum and 4 mM glutamine. Restriction enzymes (New England Biolabs), SP6 RNA polymerase (Pharmacia), RNA Guard RNase inhibitor (Pharmacia) and Streptavidin alkaline phosphatase (Boehringer-Mannheim) were used according to suppliers recommendations.

Cloning. Construction of pSFV1-NK1, which potentiates the expression of the human neurokinin 1 receptor gene, has been described earlier (16). The NK1-biot fusion was constructed as follows: The human NK1 gene was amplified with modified ends by PCR from clone pSK-NK1-9 (16). A *Cla*I site was introduced at the 5 ' end and an *Asp*718 at the 3' end. In parallel, the 1.3S biotinylatable subunit of *Proprionibacterium shermanii* (106 amino acids) was amplified by PCR with an in-phase *Asp*718 site followed by three glycine codons at the 5' terminus, and a *Cla*I site engineered immediately downstream from the translation termination site at the 3' terminus. The two fragments were ligated into the *Cla*I site of a modified pSFV1 vector, yielding pSFV1-NK1-biot plasmids. Clones with the fusion gene in the right orientation relative to the SFV 26S promoter were selected for further studies. It is noteworthy that in pSFV1-NK1 and pSFV1-NK1-biot the same 37 base pairs upstream of the NK1 receptor ATG initiation codon have been retained. This short sequence, present in the original cDNA clone, includes the natural ribosome binding sequence of the NK1 receptor gene.

Expression. *In vitro* transcripts from plasmids pSFV1-NK1, pSFV1-NK1biot, pSFV3-LacZ and pSFV-Helper2 (20) were prepared and electroporated according to Liljestrom and Garoff (21). The *in vivo* packaging was completed 24 hours after electroporation and virus stocks collected. The titer of the SFV-LacZ virus stock was determined as earlier described (16).

In expression studies confluent BHK cells were infected with the SFV-NK1, SFV-NK1-biot and SFV-LacZ virus stocks at a multiplicity of infection (MOI) of 10 and pulse-labeling carried out 16 hours post-infection. Protein expression was verified by SDS-PAGE according to Laemmli (22) followed by autoradiography or immunobiottings with streptavidin alkaline phosphatase (Boehringer-Mannheim).

Radioligand-binding assay. CHO cells were infected at a MOI of 10 on 24 well plates (1 x 10^5 cells/well) with SFV-NK1, SFV-NK1-biot and SFV-LacZ virus

stocks. The cells were prepared and analysed for [^{3}H] substance P ([Sar^9,prolyl^{2,4}-3,4(n)^{3}H] substance P) (NEN-Dupont) binding 16 hours post-infection as described by Eistetter et al., (23). The membrane fraction of infected CHO cells (5 x 10^6 cells) was isolated according to Turcatti et al., (24) and binding analysis with [^{3}H] substance P performed as above.
Intracellular calcium measurements Substance P-stimulated Ca^{2+} *mobilization with Fura-2* (1-[2-(5-Carboxy-azol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetraacetic acid) was carried out according to Eistetter et al., (23) on CHO cells 16 hours after infection with SFV-NK1, SFV-NK1-biot and SFV-LacZ virus stocks. Substance P was added at a final concentration of 10 nM. and NK1 antagonist GR147696 (1 μM) served as a control. Fluorometric determinations were carried out in a JASCO FP777 spectrofluorimeter (excitation at 340 nm, emission at 505 nm).

RESULTS AND DISCUSSION

The RNA transcribed from the pSFV-helper2 vector was used in electroporations with RNA from pSFV1-NK1, pSFV1-NK1-biot and pSFV3-LacZ, respectively. Following these coelectroporations the RNAs from both the SFV recombinant and helper were expected to be replicated and translated into proteins. This was confirmed by the pulse-labeling and immunoblotting results shown in Fig. 1. High levels of viral structural proteins, encoded by helper RNA, were detected by autoradiography 6 hours after electroporation (Fig. 1A, lanes 1 and 2). At this early stage neurokinin receptor was not clearly detected by pulse-labeling. However, the expression of the NK1-biot receptor could be visualized by the more sensitive technique of immunoblotting (Fig. 1B, lane 2).
Although the SFV recombinant and helper RNAs were both replicated in the electroporated BHK cells only the SFV recombinant RNA should be packaged

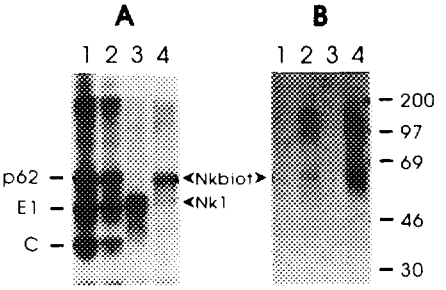

Fig. 1. *SDS-PAGE analysis of the receptors expressed in Semliki Forest virus infected BHK cells.* Protein expression was detected by [^{35}S] methionine labeling (A) and immunoblotting with streptavidin alkaline phosphatase (B) 6 hours post-electroporation and16 hours post-infection, respectively. pSFV1-NK1 + pSFV-helper2 (lane 1) and pSFV-NK1-biot + pSFV-helper2 (lane 2) electroporations; Infection with SFV-NK1 (lane 3) and SFV-NK1-biot (lane 4) virus. *p62, precursor for SFV E2 and E3* membrane proteins; E1, SFV E1 membrane protein; C, SFV capsid protein; The molecular masses of marker proteins (kDa) are indicated.

into virus particles. This is due to the packaging signal present only in the SFV recombinant RNA but lacking from the helper RNA. Therefore, infections with the viral stocks are expected to express only the genes carried by the SFV recombinant. This could be confirmed by pulse-labeling experiments (Fig. 1A, lanes 3 and 4), which showed predominant and high expression of the NK1 and NK1-biot receptors. The NK1 receptor migrates as a polypeptide of 47 kD and the NK1-biot derivative as 59 kD. This size difference is in good agreement with the 106 amino acids carried by the biotin peptide. Moreover, efficient and specific biotinylation of the NK1-biot receptor was confirmed by the results of immuno-blotting with streptavidin alkaline phosphatase (Fig. 1B, lane 4). A strong signal was observed for NK1-biot but no response seen for NK1 receptor.

Availability of radioactive substance P offered the opportunity to compare the number of receptors accessible to the natural ligand on cells expressing the NK1 receptor or its biotinylated fusion derivative. Binding was tested on whole CHO cells infected with SFV-NK1 and SFV-NK1-biot virus stocks. To ensure a complete infection a MOI of 10 was used. Saturation binding assays were conducted 16 hours post-infection (Fig. 2). From this figure number values of 1.43×10^6 and 0.93×10^6 substance P binding sites (=receptors) per cell were obtained for cells infected with SFV-NK1 and SFV-NK1-biot, respectively. In repeated binding experiments derived from infections with different SFV-NK1 and SFV-NK1-biot virus stocks, a slightly higher number of receptors per cell with SFV-NK1 was reproducibly seen. No specific [^{3}H] substance P binding was detected for cells infected with SFV-LacZ. The *K*d values of 1.2-1.4 nM is higher than observed in other studies, but may be due to the presence of low-affinity [^{3}H] substance P binding sites caused by insufficient G protein to couple to the high number of receptors being expressed.This is in good agreement with earlier observations, where increasing further the MOI for infection gave 4-6 $\times 10^6$ NK1 receptors/cell with a *K*d of 2-3 nM (17). Recently, saturation bindings on the membrane fraction of CHO cells infected with SFV-NK1 showed *K*d values of 0.13 nM which is similar to what have been observed for a stable NK1 expressing CHO cell line (Lundstrom., personal comm.)

The membrane fraction of infected CHO cells was isolated as described in Materials and methods and saturation binding assays with [^{3}H] substance P were performed as above. Again, comparable levels of receptor expression were found for SFV-NK1 (6.1 + 0.8 pmoles receptor/mg protein) and SFV-NK1-biot (3.4 + 1.3 pmoles receptor/ mg protein) in three separate experiments.

These results suggested that the added biotinylated tail did not significantly affect expression and proper NK1 binding. It seemed of interest to extend the comparison to a functionally more relevant mechanism and to see whether the tail would interfere with receptor-G protein coupling. This was amenable to analysis with the Fura-2 assay, which measures substance P-induced Ca^{2+}

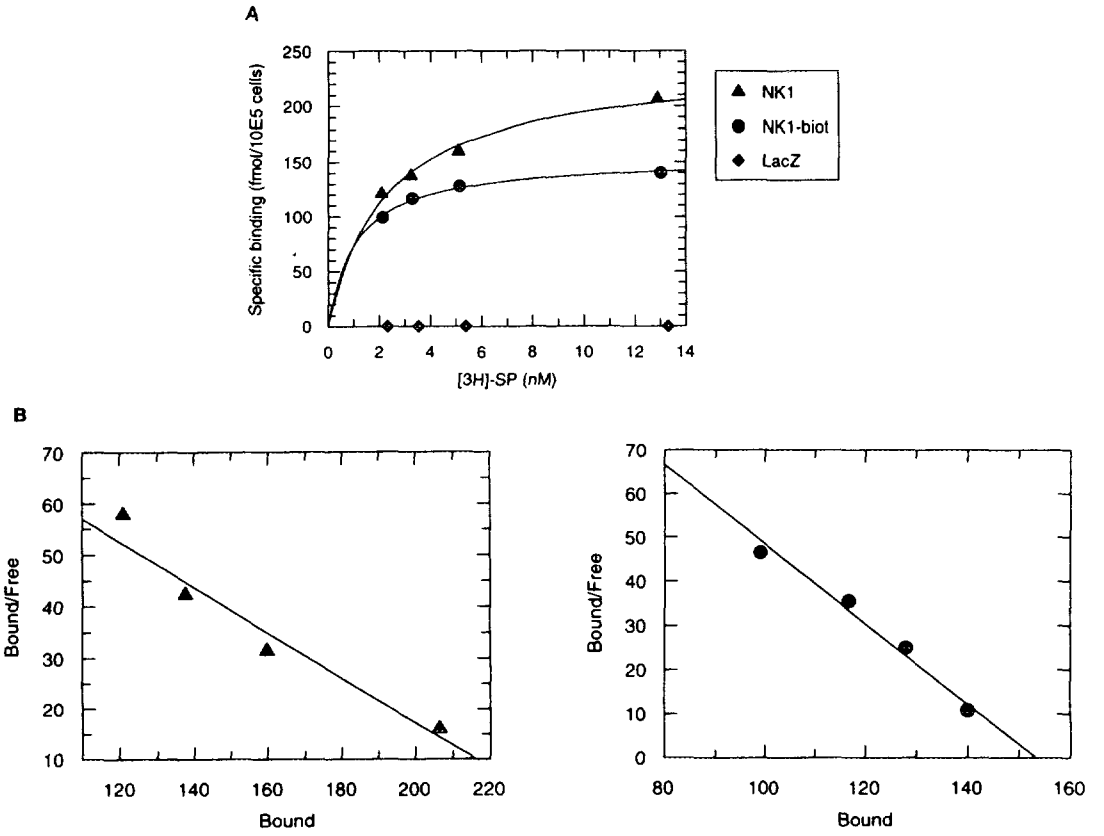

Fig. 2. A. *Saturation binding of [^{3}H] substance P to SFV infected CHO cells.* Cells were infected with SFV-NK1 (▲), SFV-NK1-biot (●) and SFV-LacZ (◆) and binding assays on whole cells carried out 16 hours post-infection. B. Scatchard plots for NK1 (▲) and NK1-biot (●).

mobilization in NK1 harboring cells. CHO cells were infected with SFV-NK1 and SFV-NK1-biot virus stocks and Fura-2 assays conducted 16 hours post-infection. Cells infected with SFV-LacZ or addition of NK1-antagonist with substance P served as controls. Similar functional responses to 10 nM substance P could be detected from both SFV-NK1 and SFV-NK1-biot infected cells (Fig. 3). By contrast, no response was observed for SFV-LacZ infected cells nor for SFV-NK1 and SFV-NK-biot infections in the presence of both substance P and the antagonist GR147696 (1 μM).

Thus by three criteria tested in the present study, i.e. expression levels, binding properties and specific signal transduction, the NK1 receptor and its biotinylated fusion derivative exhibited no significant differences. While starting the present study, our curiousity was aroused by the multiple benefits that a biotin tag may add to any protein of interest. These benefits are not restricted to facilitating protein purification, but also cover important areas such as protein detection and localization *in vivo*, or *in vitro*, protein-protein and protein-nucleic acid interaction,

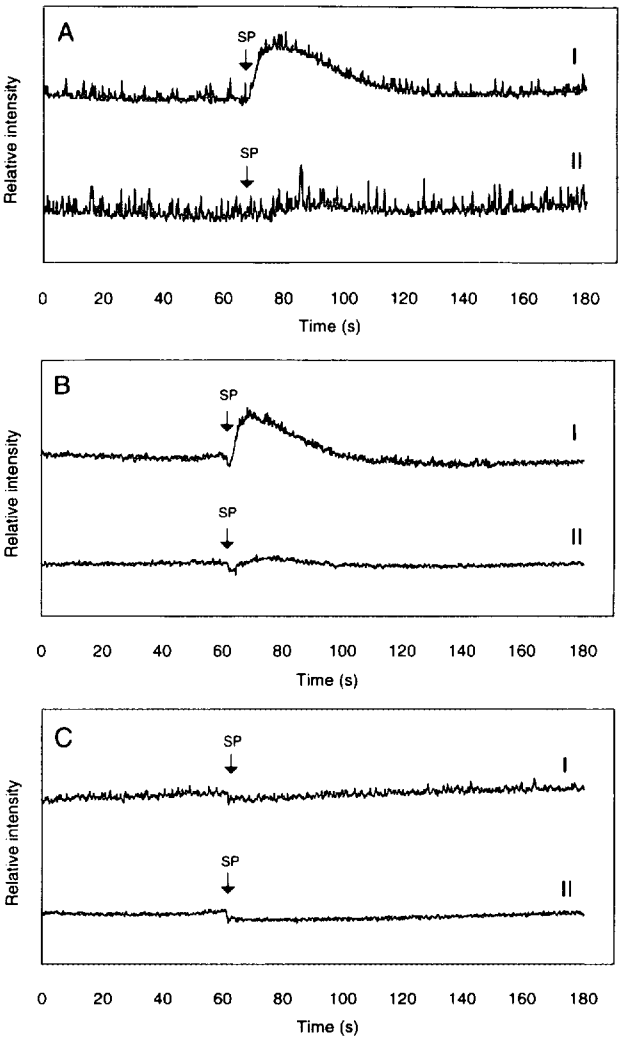

Fig. 3. *Functional response to substance P in SFV infected CHO cells.* Cells were infected with SFV-NK1 (A), SFV-NK1-biot (B) and SFV-LacZ (C) and functional activity was measured 16 hours post-infection by the Fura-2 Ca^{2+}-release assay at a concentration of 10.nM substance P (SP). I in the absence and II in the presence of 1 μM NK1 antagonist GR147696.

protein trafficking etc. For the system to be of general applicability, two requirements are needed to be met. First, the bacterial tag must also be biotinylated in non-bacterial heterologous protein expression systems. In this context, our present study confirms that mammalian cells do promote biotinylation of the bacterial tag. Incidentally, other studies in our laboratory indicate that insect cells widely used in the baculovirus expression system also biotinylate this same tag with high efficiency. The second requirement has more to do with biological significance, entailing that the altered protein must have retained native activity. This important requirement is also well fulfilled in the

present study. Without implying that it will be so for any protein engineered as described here, our results open the encouraging possibility that the biotin tag approach may prove to be a tool of choice in numerous instances.

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