

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogada
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-067268-00004-0000

Fecha: 2012-09-14 10:43:46 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 519 TRASLADOINFORME Folios: 8

ASUNTO Radicación 10-67268
 Trámite 11
 Evento 378
 Actuación 519
 Oficio
 Folios 8

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Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐

No. Solicitud Internacional PCT/EP2008/009320
Fecha de Presentación Internacional 5/Noviembre/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 64 a 118	3/Junio/2010
Reivindicaciones:	Folios 119 a 134	3/Junio/2010
Expediente 10-67268 1er Art 45		

Dibujos:	Folios 136 a 145	3/Junio/2010
Listado de Secuencias:	Folios 146 y 147	3/Junio/2010
Prioridad:	EP 07021493.7	5/Noviembre/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Conjugados de neurturina novedosas para uso farmacéutico".

El problema planteado en esta solicitud consiste en la necesidad de una variante de neurturina con una mejorada biodisponibilidad para el tratamiento de la diabetes y enfermedades neurodegenerativas.

Para resolver este problema técnico la solicitud en estudio proporciona una variante de neurturina humana conjugada con una porción de poliol.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 38/18, 47/48; A61P 3/10, 25/00; C07K 14/475.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 32 y 33 no es patentable de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

Las reivindicaciones 25 – 29 definen la composición por referencia a un uso, lo cual no es patentable, de acuerdo con el Art 14.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 – 3, 6, 8 – 13 y 30 carecen de concisión porque utilizan las expresiones “una porción de poliol”, “un producto de proteína neurturina humana”, “un fragmento biológicamente activo de la misma”, “un homólogo, ortólogo, variante, análogo, derivado, fragmento biológicamente activo o mutante biológicamente activo del mismo”, “una porción de polietilen glicol”, “una porción de polialquilen glicol”, “que tiene al menos un cambio de aminoácido en comparación con la neurturina humana”, “donde el cambio de aminoácido es entre los aminoácidos 47-69, preferiblemente 51-65 de la neurturina madura”, “donde al menos uno de los residuos de arginina (...) es suprimido o sustituido”, “donde al menos un residuo de arginina es sustituido por un aminoácido residual neutro o ácido (...)”, “donde al menos es introducido un residuo de lisina”, “variante de neurturina humana que comprende al menos uno, por ejemplo 1, 2, 3 o 4 cambios de aminoácido en comparación con la neurturina humana natural”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que un polipéptido se caracteriza por la secuencia de aminoácidos. Así mismo, una variante se caracteriza por el tipo de sustitución y la posición en que ésta ocurre. Para los términos “una porción de poliol”, “una porción de polietilen glicol”, “una porción de polialquilen glicol”, se sugiere indicar el número de unidades de poliol. Igualmente se requiere al solicitante definir a que identificador de secuencia se refieren las modificaciones de los residuos y hacer referencia a un listado de secuencias.

La reivindicación 2 no es clara cuando dice “un polipéptido codificado por la secuencia de aminoácidos expuesta en la SEQ ID NO.1”. Se sugiere al solicitante hacer la aclaración respectiva porque un polipéptido es codificado por una secuencia de polinucleótidos.

Las reivindicaciones 15 y 30 utilizan la expresión “por ejemplo” precediendo una característica, lo cual no es limitativo porque hace opcional a la característica y no limita el alcance de la reivindicación. Se sugiere incluir las características opcionales o preferentes en una reivindicación dependiente.

Las reivindicaciones 7, 23 y 24 no son claras porque utilizan las expresiones “que tiene la misma o una actividad de neurturina *in vivo* mayor que la neurturina humana nativa”, “el cual tiene una mayor biodisponibilidad del ingrediente activo en

comparación con una composición con un producto de proteína neurturina de control sin modificar”, “donde la proteína neurturina modificada está presente en una cantidad para proporcionar un incremento de al menos 2 veces, preferiblemente al menos 5 veces y de manera más preferible al menos 10 veces en la biodisponibilidad del ingrediente activo”, que son resultados a alcanzar y no definen al conjugado o la composición puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto, como la secuencia de aminoácidos.

Las reivindicaciones 13 – 29 hacen referencia a una composición. Se recuerda al solicitante que una composición se caracteriza por sus componentes y las proporciones dentro de la misma.

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Noviembre 5 de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	<u>WO2005051415</u>	Method for preventing and treating diabetes using neurturin	9/Junio/2005
D2	<u>WO9906064</u>	Method for preventing and treating hearing loss using a neurturin product	11/Febrero/1999

D1<http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2005051415&KC=&FT=E&locale=en> EP divulga un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma (Resumen, Pág. 8, líneas 26-34 y Pág. 16, líneas 3-10).

D2<http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=9906064&KC=&FT=E&locale=en> EP divulga un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma (Pág. 16, líneas 10-20 y Pág. 22, líneas 3-32).

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8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un conjugado de neurturina que comprende una porción de poliol unida covalentemente a un producto de proteína neurturina humana o un fragmento biológicamente activo de la misma.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un conjugado de neurturina que comprende una porción de poliol unida covalentemente a un producto de proteína neurturina humana o un fragmento biológicamente activo de la misma.	Un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma. (Resumen, Pág. 8, líneas 26-34 y Pág. 16, líneas 3-10)	Un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma. (Pág. 16, líneas 10-20 y Pág. 22, líneas 3-32)

Las características **esenciales** del conjugado de neurturina de la reivindicación 1 habían sido divulgadas previamente en los documentos D1 y D2 porque:

D1 divulga un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma (Resumen, Pág. 8, líneas 26-34 y Pág. 16, líneas 3-10).

D2 enseña un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma (Pág. 16, líneas 10-20 y Pág. 22, líneas 3-32).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en los documentos D1 o D2.

Las reivindicaciones dependientes 2 – 8 y las reivindicaciones 13 – 24 no mencionan alguna característica que haga nueva al conjugado de neurturina de la reivindicación

1, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La **reivindicación 9** consiste en un conjugado de neurturina según la reivindicación 8, donde el cambio de aminoácido es entre los aminoácidos 47-69, preferiblemente 51-65 de la neurturina humana madura.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Un conjugado de neurturina según la reivindicación 8, donde el cambio de aminoácido es entre los aminoácidos 47-69, preferiblemente 51-65 de la neurturina humana madura.	Un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma. (Resumen, Pág. 8, líneas 26-34 y Pág. 16, líneas 3-10)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 9 porque divulga un conjugado de neurturina que comprende una porción de poliol unida covalentemente a la proteína neurturina humana o una variante de la misma para mejorar el tiempo de circulación de la proteína (Resumen, Pág. 8, líneas 26-34 y Pág. 16, líneas 3-10). D1 también enseña que el conjugado es útil para el tratamiento de la diabetes (Pág. 6, líneas 7-12). La diferencia entre la invención, definida en la reivindicación 9 y el conjugado de D1 consiste en que el cambio de aminoácido es entre los aminoácidos 47-69, preferiblemente 51-65 de la neurturina humana madura.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja al conjugado de neurturina reclamado en esta solicitud porque no parecen presentar una mayor biodisponibilidad según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de conjugados de neurturina alternativos a los ya conocidos en D1.

Teniendo en cuenta que D1 ya divulgaba un conjugado de neurturina que comprende una porción de poliol, como polietilenglicol, unida covalentemente a la proteína neurturina humana o una variante de la misma, para mejorar el tiempo de

circulación de la proteína (Resumen, Pág. 8, líneas 26-34 y Pág. 16, líneas 3-10); además que es bien conocido en el estado del arte que las reacciones de PEGilación sobre residuos de arginina es lenta, mientras que residuos como lisina son sitios usuales para la reacción con polietilenglicol^{1,2}, y que los residuos ubicados en los dominios finger F1 y F2 son necesarios para la unión con el componente alfa del receptor GDNF³, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, utilizar técnicas conocidas en el estado del arte como mutagénesis dirigida (D1, Pág. 15, líneas 8-11) para modificar residuos de arginina dentro de la secuencia de la neurturina humana madura, pero fuera de las regiones finger F1 y F2, para obtener más conjugados con un mejorado tiempo de circulación, manteniendo la actividad de la proteína.

De manera que la solución propuesta por esta solicitud en la reivindicación 9 y en las reivindicaciones 10 – 12 es obvia y no tiene Nivel Inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	<u>WO2005051415</u>	1 – 24	Novedad y Nivel Inventivo
D2	<u>WO9906064</u>	1 – 8 y 13 – 24	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito ,

1 F. Veronese. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials, Vol.22, Pág. 405-417.
2 M. Roberts. Chemistry for peptide and protein PEGylation. Advanced Drug Delivery Reviews, Vol.54, Pág. 459-476.
3 R. Baloh. Functional Mapping of Receptor Specificity Domains of Glial Cell Line-derived Neurotrophic Factor (GDNF) Family Ligands and Production of GFRa1 RET-specific Agonists. The Journal of Biological Chemistry, Vol. 275, Pág. 3412-3420.
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Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ

Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
18 SET 2012

Elaborado por: Juan David Moncaleano P. 
Revisado por: Consuelo Leguizamón L. 

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Review

Peptide and protein PEGylation: a review of problems and solutions

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Received 8 March 2000; accepted 30 May 2000

Abstract

The paper discusses general problems in using PEG for conjugation to high or low molecular weight molecules. Methods of binding PEG to different functional groups in macromolecules is reported together with their eventual limitations. Problems encountered in conjugation, such as the evaluation of the number of PEG chains bound, the localisation of the site of conjugation in polypeptides and the procedure to direct PEGylation to the desired site in the molecule are discussed. Finally, the paper reports on more specific methods regarding reversible PEGylation, cross-linking reagents with PEG arms, PEG for enzyme solubilization in organic solvent and new polymers as alternative to PEG. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: PEG; PEGylation; PEGylation chemistry; Problems in PEGylation; Site-directed PEGylation

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1. Introduction

PEGylation is a procedure of growing interest for enhancing the therapeutic and biotechnological potential of peptides and proteins. When poly(ethylene glycol) (PEG) is properly linked to a polypeptide, it modifies

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many of its features while the main biological functions, such as enzymatic activity or receptor recognition, may be maintained. PEG conjugation masks the protein's surface and increases the molecular size of the polypeptide, thus reducing its renal ultrafiltration, preventing the approach of antibodies or antigen processing cells and reducing the degradation by proteolytic enzymes. Finally, PEG conveys to molecules its physico-chemical properties and therefore modifies also biodistribution and solubility of peptide and non-peptide drugs. PEGylation is therefore of interest in applied biotechnology because, upon modification, enzymes may become soluble and active in organic solvent. This property opens new techniques in biocatalysis and in pharmaceutical technology where many insoluble drugs are solubilized by PEG conjugation and thus more easily administered [1].

Several authors reviewed different aspects of PEGylation with emphasis on the polypeptide and protein conjugation and also on the modification of liposomes, particles and non-peptide drugs [2–9]. This article provides a general survey of the PEGylation technology, with special emphasis being given to contributions for the solution of specific problems encountered during PEG conjugation.

2. Problems in PEGylation related to the starting PEG

The unusual properties that PEG conveys to proteins such as solubility, biocompatibility, protein rejection, and modification of pharmacokinetics, to name only a few, are the “reason d'être” of this technology. However, PEG, being a synthetic polymer, is polydispersed and, even in the best of cases, a polydispersity value (M_w/M_n) ranging approximately from 1.01 for low molecular weight oligomer (3–5 kDa), to 1.2 for high molecular weight (20 kDa) may be expected. This polydispersity is a negative property since it is reflected in polydispersity of the conjugates. At present, the best way for testing the M_w/M_n ratio is MALDI mass spectroscopy (Fig. 1), but GPC with a refractive index detector is also useful.

The monomethoxylated form of PEG is generally used in protein conjugation, since its monofunctionality yields cleaner chemistry. However, a certain amount of PEG diol is always present, in the range of 1–10%, depending upon the molecular weight (lower for low-mass PEG). The level of polydispersity may vary greatly among the different suppliers and, as a general rule, we would recommend to evaluate product homogeneity before its application by using one of the techniques mentioned above. In the case of GPC a peak or shoulder in front of the main elution peak is observed, corresponding to the diol by-product, which is characterised by a molecular weight approximately double that of the mono-functional methoxy-PEG. High diol concentration will yield unwanted cross-linked conjugates.

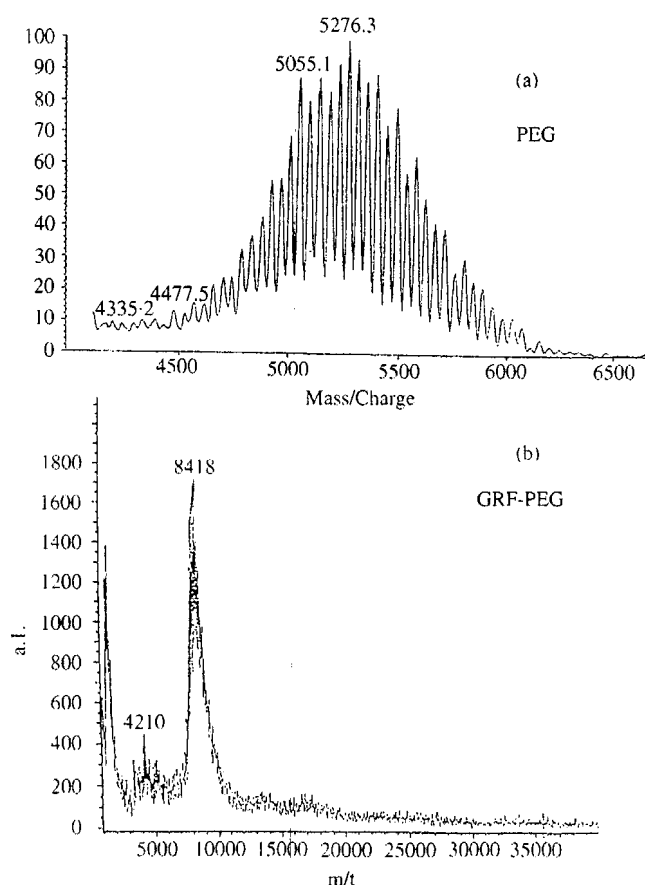


Fig. 1. PEG polydispersity: (a) polydispersity of PEG of 5 kDa as assessed by MALDI mass spectrometry and (b) mass spectrum of the conjugate of this PEG species with the growth hormone releasing factor, a peptide of 3358 kDa. A broad peak centred at 8418, corresponding to the sum of PEG and peptide masses, is obtained. The polydispersity of PEG is reflected into the polydispersity of the conjugate.

PEG molecular weight has a great influence on the biological properties of conjugates. The data reported in Table 1 on the residence time of SOD and SOD conjugates of different mass demonstrate this point.

3. Activated PEGs for amino group conjugation

Since most applications of PEG conjugation involve labile molecules, the coupling reaction generally requires mild chemical conditions. In case of polypeptides, the most common reactive groups involved in coupling are nucleophiles with the following decreasing rank order of reactivity: thiol, alpha amino group, epsilon amino group, carboxylate, hydroxylate. However, this order is not absolute, since it depends also on the reaction pH, furthermore other residues may react in special conditions, as the imidazole group of histidine.

The thiol group is rarely present in proteins, furthermore it is often involved in active sites. The carboxylic groups cannot be easily activated without having

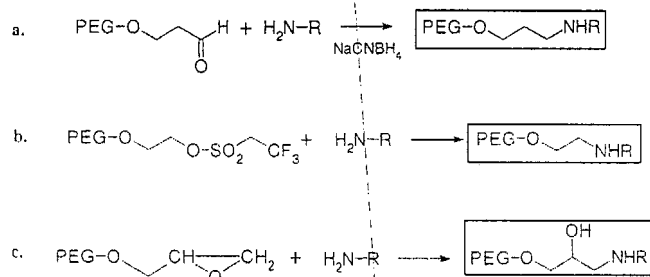


Fig. 2. *Alkylating PEGs*: (a) PEG aldehyde: Best reaction takes place with α - and ϵ -amino groups in protein at neutral or mild alkaline pHs, while good selectivity for the α -amino terminal amino acid at pH 5–6, was found. (b) Trisylated-PEG: Best reaction takes place with amines around pH 8 and low temperature. The positive amino charge is maintained. The reaction rate is depending upon the properties of the peptide, but doubts remain on the structure of the reaction products. (c) PEG epoxide: Reaction at high pH, 8.5–9.5, is preferred for amino conjugation, although binding to hydroxyl, imidazole and thiol groups also may take place.

reaction with the protein amino groups, to yield intra or inter molecular cross linking. Therefore, amino groups, namely the alpha amino or the epsilon amino of lysine, are the usual sites of PEG linking. As a consequence, the problem is to transform the hydroxyl terminal group of PEG to an activated one. In the literature more than a dozen of such procedures are described and for a detailed discussion, the reader is referred to excellent reviews and, among these, the ones by Schacht or Zalipsky [4,5,7].

For a general survey, these activated PEGs may be divided into two important classes.

3.1. Alkylating PEGs

These products do not modify the charge of amino residues. Among these stands *PEG-aldehyde* that gives a permanent linkage after Schiff base formation followed by cyanoborohydride reduction (Fig. 2a) [10]. This is a convenient way for conjugation when the amino positive charge is critical for the retention of biological activity. However, the reaction rate for the Schiff base formation is relatively low, sometimes up to a day is necessary to reach the completion, with consequent inactivation of labile molecules. The reaction pH is critical for selectivity, α -amino terminal modification can be achieved around pH 5 [11]. *PEG-trisyl chloride* activation is an alternative method to maintain positive charge [12] (Fig. 2b). However, the chemistry of conjugation and the conjugation products are not unique and well defined [12–14]. The use of *epoxy PEG* has been used, but again, as with PEG-aldehyde, the reactivity is low and furthermore the specificity is not certain since hydroxy groups may also react (Fig. 2c).

3.2. Acylating PEGs

Most of these are *hydroxysuccinimidyl esters* (–OSu) of carboxylated PEGs (Fig. 3a). It is important to know

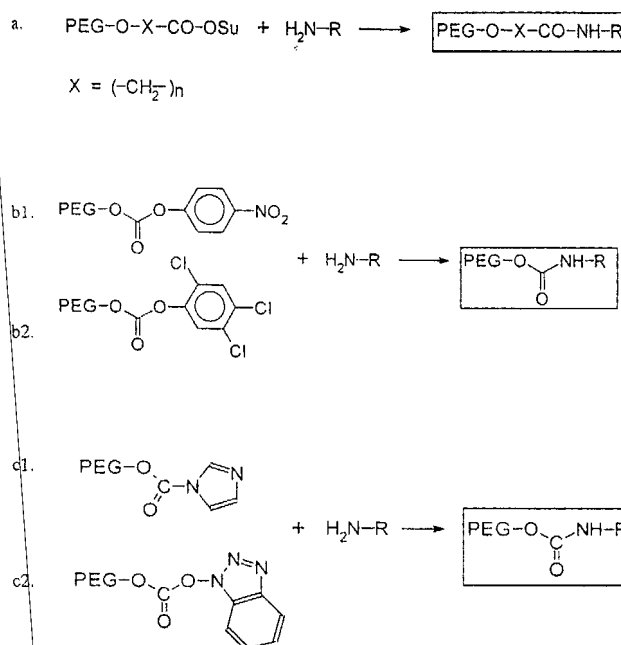


Fig. 3. *Acylating PEGs*: (a) Activated alkyl acids: These compounds are highly reactive towards amino groups, but the reaction rate may change significantly from the X structure. (b) PEG-*p*-nitrophenylcarbonate (b1) and PEG-trichlorophenylcarbonate (b2): The reaction rate is much slower of the OSu activated carboxylate-PEG. Carbamate derivatives are obtained. (c) PEG-oxycarbonylimidazole (c1) and PEG-benzotriazole carbonate (c2): Both reagents yield carbamate linkages with amino groups, but with different reaction rates.

that the distance between the active ester (–COOSu) and the last PEG ether can vary in different available products, by up to four methylene units, and this has profound influence on the reaction towards amino groups as well towards water. As an example, the $t^{1/2}$ hydrolysis rate of PEG-O-CH₂-CH₂-CH₂-COOSu is 23 h, while that of PEG-O-CH₂-COOSu is 0.75 h [15].

In another acylating chemistry the terminal PEG hydroxy group is activated by chloroformates or carbonylimidazole. We have special experience with the first ones, namely with *PEG-p-nitrophenylcarbonate* or *PEG-trichlorophenylcarbonate* [16] (Fig. 3b). These reagents exhibit lower reaction rate than the –OSu activated PEGs and this property allows exploitation of different reactivity among the amino groups of the macromolecule by stopping the reaction at the desired time of modification. In this way some selectivity in the conjugation based on amino group availability can be achieved. Furthermore, reactions in aqueous-organic solvents are feasible. There is an interesting difference between these two carbonate-activated PEGs: the first one has a pale yellow colour, whose intensity increases with the conjugation reaction for the formation of phenol as a leaving group, while the second is a colorless product. The extent of amino groups modification can be followed in the case of the second product by colorimetric analysis to titrate the residual amino group directly on aliquots of the reaction

mixture without the need for removing excess reagent or reaction by-products [17]. If the fluorimetric method, based on the reaction of fluorescamine, is used, the degree of amine modification can be evaluated using a much lower amount of product [18]. A urethane linkage with amino groups is also obtained with other electrophilically activated PEGs. Among these are *PEG-oxycarbonylimidazole* [19] and *PEG-benzotriazole carbonate* [20] (Fig. 3c). The first one is characterised by a lower reaction rate as compared to chloroformates, while the second one is very reactive, although less so than the succinimidyl activated carboxyl PEGs.

4. PEG with amino acid arm

The presence of an amino acid or peptide arm between PEG and the attached macromolecule gives several

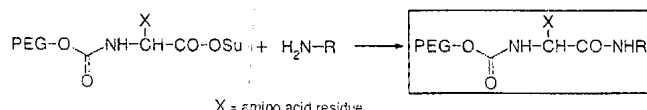


Fig. 4. *PEG amino acids*: A variety of properties may be conveyed to the PEG-conjugate using different amino acids or peptides. Examples may be: Nle for analytical purposes, ^{14}C or tritium labelled Gly for pharmacokinetic studies. Trp for fluorescence labelling. Lys for branching. Met-Nle or Met- β Ala for PEG removal by BrCN treatment.

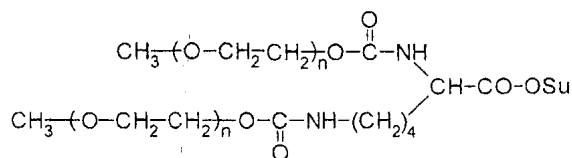


Fig. 5. *Branched PEG (PEG2)*: Lysine is the amino acid spacer between two PEG molecules and the activated carboxylate. This PEG offers the advantages of higher protection of protein surface for single point of conjugation, as compared to the linear form. Generally, it conveys to its products higher retention in blood, lower immunogenicity and decreased enzyme inactivation during the conjugation.

advantages due to the variability of properties that may be introduced using a suitable amino acid or peptide. For the convenience of synthesis the amino acid or peptide can be linked through its amino group by urethane linkage to PEG, while the carboxyl group, properly activated, is bound to the amine of the macromolecule [21] (Fig. 4). Among these arms, norleucine is of interest for the advantage in analysis of PEGylated proteins [22]. A second is the so-called branched PEG or PEG2 [23]. This PEG derivative is characterized by two linear PEG chains linked together through two functions of a tri-functional spacer, while the third function is used to bind the protein. Lysine is the tri-functional amino acid spacer and the two PEG chains are linked to its alpha and epsilon amino groups while the carboxylic group is activated as -OSu for protein binding (Fig. 5). This PEG has the advantage of a lower inactivation of the enzymes during conjugation (see below). Furthermore, and more important, for its “umbrella-like” structure it is more effective also in protecting proteins from proteolysis, in the approach of antibodies, and in reducing immunogenicity [24,25] (Fig. 6).

5. Characterization of PEG conjugates

The observations reported above on the degree of modification obtained by the colorimetric titration of the non-conjugated amino groups, opens the general problem of the precise evaluation of the number of polymer chains bound to peptide or proteins. PEG is transparent and non-fluorescent and therefore non-detectable by itself, furthermore it does not release products easy to quantify upon hydrolysis. The colorimetric reaction of PEG with iodine can be used for its direct evaluation [26] but it possess low-sensitivity relative to the high values of the blanks. The different retention time in GPC elution, shown by differently modified conjugates, was also suggested as a tool for the evaluation of the degree of

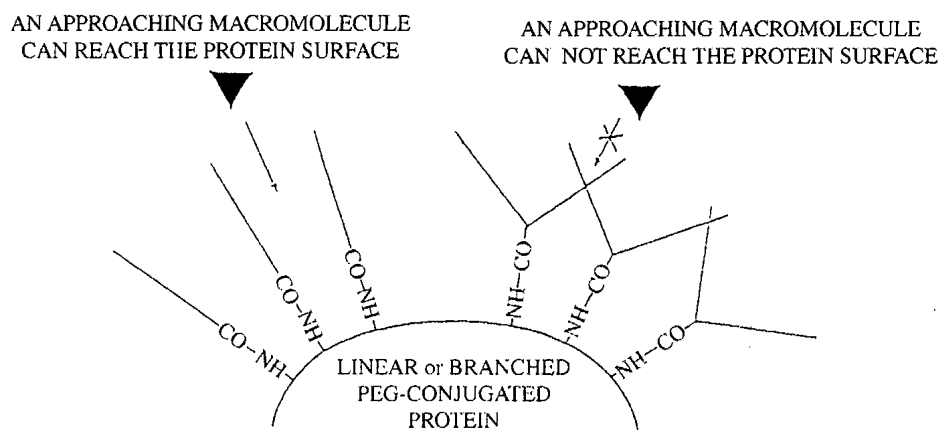


Fig. 6. The “umbrella-like” structure of branched PEG explains the higher capacity in rejecting approaching molecules or cells as compared to linear PEG of the same size.

polymer substitution. However, the elution time in GPC is not directly related to the increase in molecular weight due to the linked PEG, since the hydrodynamic volumes of PEG and the protein are very different, the first being higher by about two fold of that of a protein with the same molecular weight.

Usually, the number of PEG-bound chains is calculated indirectly from the decrease of unreacted amino groups, with respect to the total present before the reaction, by using the colorimetric or fluorimetric methods as reported above. However, these methods may not always give reliable results, due to the intrinsic variability of any colorimetric or fluorimetric determination and to the contemporaneous need for a separate evaluation of protein concentration.

As an alternative, MALDI mass spectrometry is now frequently employed because it yields a complete picture of the mixture of components of the conjugation reaction based on the mass (Fig. 7). However, in this case, the method is not quantitative, due to the different extraction

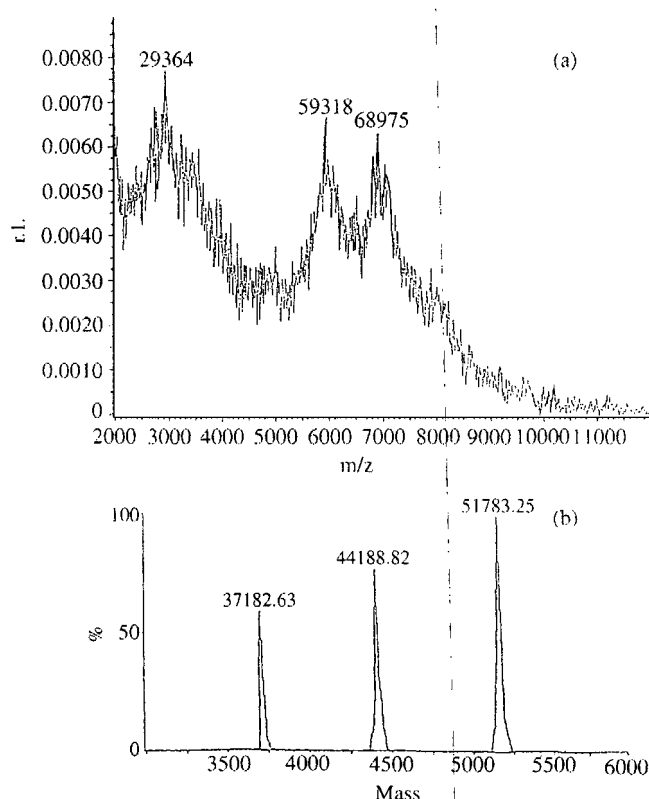


Fig. 7. (a) Maldi mass spectrometry of a subtilisin (MW 29 kDa) PEGylated with branched PEG 10 kDa. Two major peaks of equal area are present corresponding to subtilisine conjugated with 3 and 4 branched PEG molecules and a minor peak with 5 PEG molecules. The first large peak (centred at 29.3 kDa) refers to the conjugate with multiple charges. To note that the amount of the conjugates with 3 and 4 PEG chains, that appears equal in this spectrum, when evaluated by GPC chromatography was found to be largely different, a ratio of approximately 1 : 2. (b) Electrospray mass spectrometry of PEG-subtilisine after deconvolution. The presence of conjugates with 2, 3 and 5 PEG chains is shown (from Giacchini AM et al. [84]).

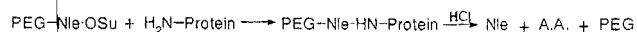


Fig. 8. *PEG-norleucine*: This PEG is useful for absolute evaluation of the number of PEG chains bound to a protein. This number is obtained from Nle by amino acid analysis after acid hydrolysis.

yield from the matrix of compounds of different weight. Electrospray mass spectrometry was also proposed for the evaluation of poly(ethylene glycol)-protein conjugates. Subtilisine and lipase and PEG 5000 Da were the first models successfully investigated by this technique [29]. The advantage of both these mass spectrometric methods resides in the fact that they can be used with any of the PEG coupling methods employed: acylating or alkylating, involving amino, thiol, carboxylic or arginine groups.

In order to overcome the problems connected with the evaluation of the degree of polypeptide modification, a procedure was devised that does not yield the pattern of modified products as mass spectrometry does (for this a preliminary chromatographic step would be necessary), but it gives the overall modification extent of the conjugation product [22]. The method exploits the use, as a spacer arm between PEG and protein, of the amino acid norleucine (which is absent in proteins) activated as -OSu at its carboxylic group. The PEG-Nle conjugated protein is hydrolysed by HCl and amino acid analysis is carried out according to a standard procedure: the number of bound PEG chains corresponds to the number of Nle. A stable amino acid is used as internal reference standard to calculate the ratio between Nle and protein (Fig. 8).

Capillary electrophoresis has also being reported for protein PEG-conjugate characterization. This method allows a wonderful separation of PEG-protein isomers that cannot be easily reached by HPLC or by ion-exchange chromatography [27,28]. Finally, SDS electrophoresis may also be of use but its success is limited to PEGylated peptides or low molecular weight proteins since the great hindrance of the conjugates interferes with the penetration inside the gel.

6. Unexpected reaction in PEGylation chemistry

To get a precise characterization of PEG conjugates more than a single analytical method must be employed. A typical example of this need comes from a recent study carried out in our laboratory for the modification of a genetic variant of epidermal growth factor (EGF) [30]. In this case, using the colorimetric procedure of Habeeb [17] to evaluate the number of bound polymer chains, we could not realise that PEG was bound to the peptide at the level of a tyrosine residue in addition to two amino groups.

This tyrosine conjugation, unexpected for a PEG activated as hydroxysuccinimidylester, could in fact be revealed only by MALDI mass spectrometry or by the

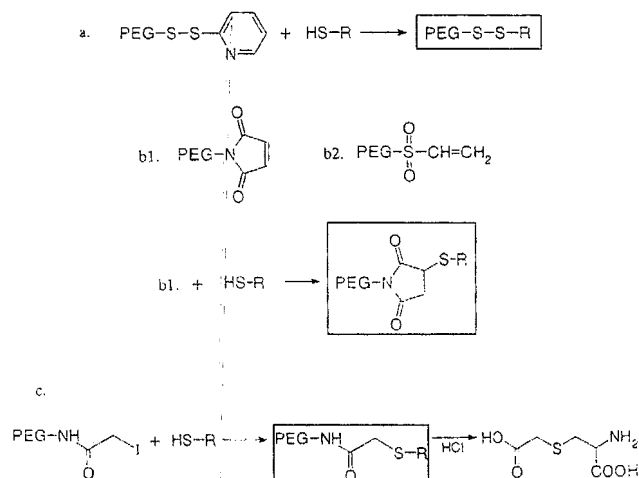


Fig. 9. Thiol reactive PEGs: (a) PEG-ortho-pyridyl-disulphide: The reaction conjugate with this PEG may be quantitatively reversed by thiol treatment. (b) PEG-maleimide (b1) and PEG-vinylsulphone(b2): The thiols give stable addition to the double bond of these PEGs. The reaction rate is pH dependent, best conditions being around 8. To note that the amine addition is not excluded specially increasing the pH or when organic cosolvents are present. (c) PEG-iodoacetamide: A stable thioether is formed in mild basic media. By strong acid hydrolysis carboxymethylcysteine (CMC) is quantitatively obtained from the cysteine derivative. CMC may be evaluated by standard amino acid analysis thus offering a method to assess the cysteine conjugation.

norleucine procedure of analysis described above, which showed an additional PEG chain. This extra chain could be removed from the conjugate by mild hydrolysis, as expected for an ester linkage. Tyrosine modification in the EGF molecule was probably related to the special amino acid arrangement in the sequence of this polypeptide, since it did not take place in the homologous human protein.

7. PEGylation at cysteine residue

Few proteins possess thiols suitable for PEG binding. However, this rare residue may be introduced at the desired position of the sequence by genetic engineering, a strategy that offers the conditions for site-directed PEGylation. This sequence-specific modification may take advantage of the few thiol reactive PEGs. Among these there is an activated disulphide, namely *PEG-ortho-pyridyl-disulphide* [31], that reacts with thiols yielding the more stable symmetric disulphide (Fig. 9a). A second reagent, *PEG-maleimide*, takes advantage of thiol addition to the activated double bond, known from many years in protein chemistry as the Michael reaction [32]; a new reagent, based on thiol addition to *PEG-vinylsulphone* double bond was devised in a joint collaboration between our laboratory and Prof. M. Harris in Huntsville [33] (Fig. 9b). Both of these last two reagents react with the sulphydryl group only

at neutral or mild alkaline pH (7.5–8.5) while, when the pH is increased, the amino group addition may also take place, although with significantly lower kinetics. It may be interesting to note that this unwanted amino reaction is enhanced by the presence of an organic co-solvent in the reaction mixture [34]. *PEG-iodoacetamide* was also employed (Fig. 9c) as a well-known reaction in protein chemistry. This modification procedure presents the advantage that, by strong acid hydrolysis, the PEGylated cysteine gives rise to carboxymethylcysteine, a stable cysteine derivative that can be identified and quantified by standard amino acid analysis [35].

8. Arginine modification

Only few examples of polypeptide PEGylation at the level of arginine were reported so far, mainly in the patent literature, all based on the use of *PEG-1-3-dioxocompounds* (Fig. 10). The disadvantage of this method is related to the long reaction time needed for complete coupling that may be harmful to the protein stability, and to the non-specificity of its chemistry since other amino acids, histidine and lysine in particular, may also react [36,37].

9. Carboxyl group modification

Amino PEG may be used to PEGylate carboxyl groups, by the use of *N,N'*-dicyclohexylcarbodiimide or a water-soluble coupling agent as *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride. However, the procedure is successful only when amines are not present in the compound, as for instance in the case of non-peptide drugs. In peptides and proteins the risk of cross-linking is difficult to avoid.

An original strategy was devised by Zalipsky to specifically PEGylate carboxylic groups in proteins without cross-linking formation with amino groups: it takes advantage of the linkage of PEG to carboxyl groups only, by the use of *PEG-hydrazide* [38] (Fig. 11). The very low pK of the hydrazide group allows, in the presence of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, its binding to carboxyl groups in acid media (pH 4.5–5), where all the protein amino groups are protonated and not reactive. This condition assures that intra- or inter-molecular protein cross linking between carboxylates and a PEG amine takes place.

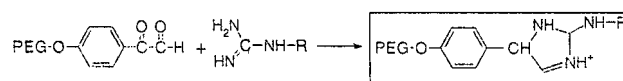


Fig. 10. PEG-phenylglyoxale: The reaction rate of this PEG with arginine is low and other amino acids are modified as well.

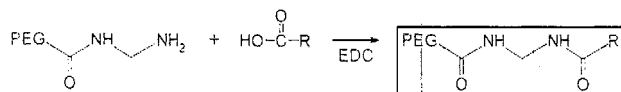


Fig. 11. *PEG-hydrazide*: At pH 4–5.5 only the conjugation between PEG-hydrazide and the carboxylic groups takes place, because under such pHs the protein or peptide amino groups are protonated and not available for binding.

10. Hydroxyl group conjugation

PEG-isocyanate is useful for hydroxyl group conjugation yielding a stable urethane linkage [39]. However, its reactivity may be best exploited for non-peptide moieties such as drugs or hydroxyl-containing matrices for chromatography and also to yield biocompatible surfaces. PEG-isocyanate is in fact highly reactive with amines also (Fig. 12).

11. Active site protection from PEGylation

The same mechanism that prevents the approach of proteolytic enzymes or antibodies to PEGylated protein can also reject a substrate from the protein active site. This mainly occurs with enzymes possessing high molecular weight substrates such as peptides, proteins, polysaccharides, and also in the receptor-binding of PEGylated molecules. In both cases this effect prevents or largely reduces the advantages of PEG conjugation technology. Methods that can be exploited under different circumstances were devised to overcome this problem. These are summarised as follows:

1. In a few cases, the problem was reduced by carrying out the conjugation in the presence of an active-site protecting agent, such as a substrate, an inhibitor, or other agent with specific affinity for the macromolecule. Protection of the binding site from PEG conjugation was observed to some extent [40]. However, the results, using this approach, are generally unsatisfactory because PEGylation may still occur in the surroundings of the protected site.

With this in mind, a procedure was devised, based on the use of an inhibitor (as a benzamidine derivative in case of serine protease) covalently linked to an insoluble resin (agarose) that, at proper pH and ionic strength binds reversibly, by affinity, to the enzyme and protects both the active site and its close surroundings from reacting with PEG. After the removal of the insoluble inhibitor by changing pH, the enzyme was found to maintain activity also towards high molecular weight substrates such as albumin in case of trypsin, or blood clots in the case of urokinase [41,42] (Fig. 13). In another case the activity of proteolytic enzymes was instead maintained by the use of very large polypeptide inhibi-

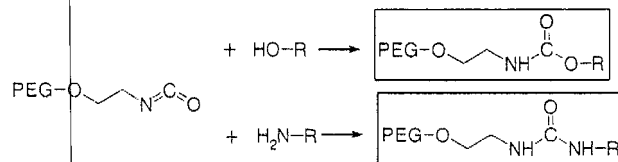


Fig. 12. *PEG-isocyanate*: This PEG is mostly used for PEGylation of polysaccharide gels or for surface modification. Attention must be paid to the presence of dimers.

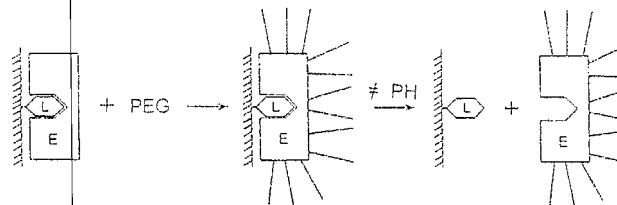


Fig. 13. A ligand, such as a substrate or inhibitor (L), linked to an insoluble matrix gives reversible binding with the enzyme or protein and prevents the PEGylation around the binding site. The PEGylated macromolecule is released from the ligand by any condition that reverses the binding such as pH, salts, solvents.

tors (as kunitz inhibitor) added in solution to the conjugation mixture [43]. Also in this case the protecting agent was removed, at the end of conjugation reaction, by changing the pH to a value that minimizes non-covalent binding forces.

2. The problem of loss of biological activity during conjugation may be circumvented by exploiting a different structure in the polymer used for modification. In this regard, observations are of interest with some enzymes, uricase or asparaginase as typical examples. It was found that the activity loss of these enzymes was largely prevented by using a special form of PEG, called branched PEG or PEG2 (see above). Using this PEG, enzyme activity is preserved to a larger extent as compared to linear PEG, most probably because polymer approaching to the active site cleft is hindered by its bulky structure (Fig. 14). As in the case of uricase conjugation, the residual PEGylated enzyme activity is 32% of the starting one, if a branched PEG is employed for the conjugation, but 2.5% only if a linear PEG of the same mass is used [25].

The same mechanism limits also the sites of PEGylation in the polypeptide allowing a conjugation, in the case of the branched form, to the more accessible amino acid residues only. This is well demonstrated in α -interferon conjugation that, once modified with the linear polymer, gave rise to eleven positional isomers, whereas, with the branched polymer, only one or two lysine residues were sites of PEGylation [45–47].

3. In case of smaller macromolecules, such as peptide hormones or cytokines, activity loss may be prevented by using specific chemical strategies aiming to tailor PEGylation at the level of non-essential residues. This may be reached taking advantage of different residues reactivity due to solvent exposure or nucleophilicity.

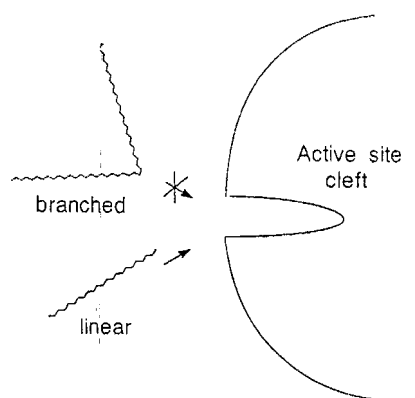


Fig. 14. The high steric hindrance of branched PEG may be advocated to explain the lower inactivation of enzymes as compared to linear PEG of the same size.

4. If nature does not offer the lucky situation reported above, another possibility is to carry out the modification in a stoichiometric deficiency of PEG to obtain isomers that can be separated later by chromatography. Each isolated isomer is assayed for biological activity so that the most promising one can be selected for further development. This strategy was followed in our laboratory with the 1–29 sequence of the growth hormone releasing factor that, having three reactive amino groups, could lead to seven potential conjugation products: three mono-PEGylated isomers, three di-PEGylated and one tri-PEGylated form. In this case, however, this number was drastically reduced because of a low reactivity of the alpha amino residue and, when the reaction was carried out in stoichiometric deficiency of PEG, two mono-PEGylated isomers only (in positions 12 and 21, respectively) were obtained as the main products. One PEG isomer proved to be the most biologically active, and a procedure for its convenient isolation was optimised [44]. A similar strategy has been used for the modification of alpha-interferon where eight mono-substituted isomers were obtained and, after purification to homogeneity, assayed for a structure activity relationship [45]. A further successful example of separation between mono and di-PEGylated products was reported in salmon calcitonin conjugation by PEG 12 kDa. In this case it could be demonstrated that the conjugated species were equally bioactive as the native peptide [48].

5. A more sophisticated strategy to reach specific PEGylation is based on a preliminary chemical reversible site-directed protection of the peptide to leave some groups free for PEG conjugation. This was the case of insulin modification since in alkaline conditions glycine A1 and lysine B29 were preferentially protected by BOC to PEGylate Phe B1 only [49–51]. Further example is the reversible protection of the alpha or the epsilon amino groups of a somatostatin analogue achieved carrying out the reaction at different pHs with a suitable reagent [52].

6. A quite different approach was instead followed by Campbell [53], who obtained GRF-PEGylated peptides during the synthetic step by using PEGylated amino acids.

12. PEG as flexible, hydrophilic moiety in bifunctional reagents

Bifunctional reagents are a major interest in biology or applied sciences, and a list of reagents with different chemical properties were described and reviewed [54]. The placing of a PEG chain between two reactive moieties extends the application of cross-linking reagents where hydrophilicity, flexibility and biocompatibility are needed. Furthermore, the variety of PEG length available in the market allows tailoring of the distance between the two reactive entities and cross-linking macromolecules when the reaction would be hampered by steric hindrance. New applications are in the preparation of supports for solid or liquid phase peptide synthesis [55,56], targetable polymeric drugs [57], linking mobile cofactors to apoenzymes [58], grafting on surfaces and conjugating fluorescence probes to macromolecules. For bifunctional PEG preparation a diol PEG of desired molecular length is the starting polymer, while the two terminal hydroxyls are activated with the same reactive group or with groups of different reactivity. In the last case, since amino and thiols are the residues most reactive in macromolecules, α -OSu-activated carboxylic groups on one side and vinyl sulphone, maleimide or the pyridyl disulfide on the other, are the bifunctional PEG preferred end groups. This choice allows carrying out the cross-linking in a sequential order thanks to the different reaction conditions of the two groups (Fig. 15).

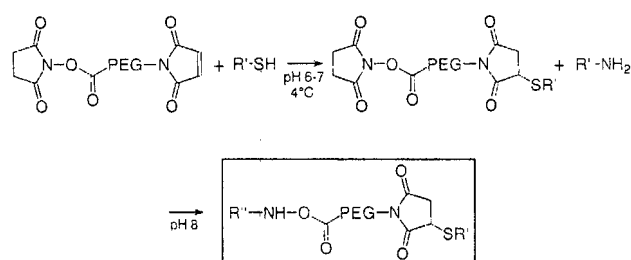


Fig. 15. α -OSu-O-maleimide-PEG: This is a typical cross-linking reagent that may react in a sequential order with different groups in protein, taking advantage of different pH and temperatures. With this strategy two different macromolecules may be stoichiometrically linked together.

13. Enzyme PEGylation for solubilization in organic solvents

Enzymes are becoming new tools for specific bioconversions in organic synthesis. However, they suffer from

insolubility in most solvents where many compounds of pharmaceutical and cosmetic interest may be dissolved. Poly(ethylene glycol) chains bound to the enzymes overcome such limitation because they convey organic solvent solubility without impairing, in many instances, enzymatic activity [59]. For this purpose, enzyme PEGylation may be carried out with any of the above reported methods. Most used, however, is the one that takes advantage of trichlorotriazine (*PEG-dichlorotriazine*), since it is easy to prepare, gives stable products with both amino and thiol groups and its chemistry is well known [60]. The risk of toxicity of the intermediate of this reaction, that is of concern in therapeutic enzymes, does not hold for this application as well as the possibility of cross-linking. A branched form of PEG-triazine was also prepared that exploits two chlorides of trichlorotriazine for PEG binding while the third is used for enzyme conjugation [61] (Fig. 16).

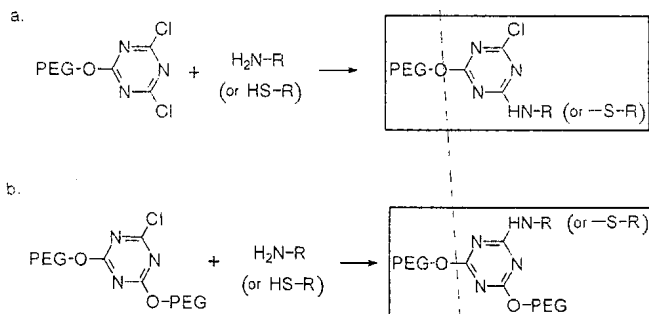


Fig. 16. *PEG-triazine*: Linear (a) or branched (b) PEG based on trichlorotriazine activating moiety. The linear form may yield cross-linked products for the presence of the remaining chloride in the triazine ring.

14. Reversible grafting of PEG to proteins or fatty acid

In several instances it may be useful to selectively remove PEG from its conjugates. Some strategies were reported that take advantage of the introduction of labile bonds between PEG and the conjugated moiety. One of these exploits the use of maleic acid between PEG and protein. A cleavage takes place in mild acid medium at room temperature thanks to the anchimeric assistance of the carboxylate, enhanced by the presence of a double bond, to the nearby amide bond [62] (Fig. 17). In two further reports cleavage is achieved by thiols: in the first

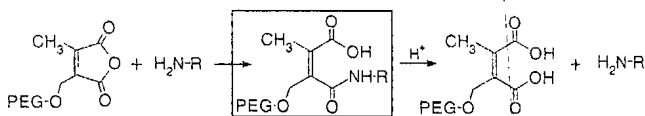


Fig. 17. *PEG-maleic-anhydride*: The PEGylation may be reversed by anchimeric assistance of the neighbouring free carboxyl function which is facilitated by the presence of the close double bond.

the conjugating moiety is an aliphatic disulphide, namely 3,3'-dithiodipropionate that is cleaved by 1,4-dithiothreitol treatment [63]; in the second the conjugating moiety is a thiophenyl group that releases PEG from distearoylphosphatidyl ethanolamine by mild thiol treatment [64].

15. Identification of the PEGylation site in peptide and protein

While with small peptides the exact location of the PEG chain can be easily identified, because the Edman degradation may be carried out on the conjugate, this is very difficult or impossible with larger conjugates. Note also that in the stepwise degradation of a conjugate the PEGylated site is identified by a missing amino acid. On the other hand, in proteins, Edman degradation may be carried out only after cleavage to smaller peptides. In this case, a problem resides in the difficulty to isolate and purify the individual products of the digestion, as often the polymer chain makes them equivalent in properties. Furthermore, the hindrance of the bound polymer chains may prevent the specific cleavage by proteolytic enzymes needed in sequence studies. For these reasons few investigations of sequence characterization in PEGylated proteins have so far been reported.

A successful exception concerns the characterization of a PEGylated α -interferon mixture, obtained in stoichiometric deficiency of PEG as reported previously, in which the monoPEGylated isomers were separated by ion-exchange chromatography and each peptide identified by Edman degradation [45]. In a more complex case, poly-PEGylated growth hormone was hydrolysed by trypsin and the site of modification identified by the indirect (and dubious) evidence based on the absence of the expected tryptic peptides as compared to the digestion of the native growth hormone [65]. Incomplete proteolysis is in fact expected at the level of PEG.

These difficulties may be greatly reduced by a recent method that takes advantage of the properties of two special PEGs with methionine in the peptide arm, PEG-Met-Nle or PEG-Met- β Ala [66] (Fig. 18). The presence

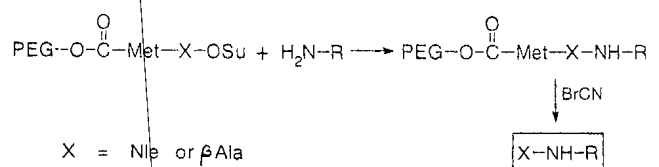


Fig. 18. *PEG-Met-Nle-OSu* and *PEG-Met- β Ala-OSu*: PEG protein conjugation, and PEG release by BrCN treatment, leaving Nle or β Ala as reporter groups bound to protein at the residues where the polymer was present.

of the methionine allows the removal of the polymer chains from the protein molecule by cyanogen bromide treatment following a reaction well known in protein chemistry. After cleavage at the level of methionine, nor-leucine in the first case, or β -alanine in the second, remains linked to the protein as a reporter group in the position of the PEG chains. The proteins, now devoid of the bulky polymer, may be more easily fractionated into the position isomers than the PEGylated form and the different components analyzed by any sequence procedure. The protein or the peptides obtained by digestion, with linked unnatural amino acid Nle or β -Ala, may also be analysed by MALDI or electrospray mass spectrometry to reveal the eventual increase in weight due to this new amino acid, or characterised by amino acid analysis after acid hydrolysis or sequenced by Edman degradation. The procedure described here was successfully tested in our laboratory using as models lysozyme, a decapeptide partial sequence of glucagone, and insulin with PEG linked in different positions [67,85].

16. New monofunctional polymers for protein conjugation

The basic reason for the success of methoxypoly(ethylene glycol) among other polymers resides in its monofunctionality. This prevents the formation of cross-linked conjugates that would take place with polyfunctional polymers as polysaccharides, most of polyacrylates or serum albumin, all reagents already proposed for conjugation [68–71].

To extend the potential of peptide and protein conjugation, new monofunctional oligomers were investigated that convey different properties to the conjugates. Among these are *poly(N-acryloylmorpholine)* (PACM) (a) and *poly(N-vinylpyrrolidone)* (PVP) (b) (Fig. 19). They are obtained by a special polymerization process that yields oligomers with the desired molecular weight (in the range of 3000–6000 Da) and with a single terminating functional group [72,73].

The studies carried out so far with these two polymers using superoxide dismutase, ribonuclease, uricase and

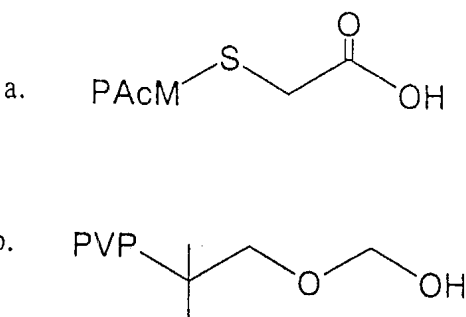


Fig. 19. Carboxyl (a) or hydroxyl (b) terminating *poly(N-acryloylmorpholine)* (PACM) and *poly(N-vinylpyrrolidone)* (PVP) as alternative polymers to PEG for protein or liposomes modification.

asparaginase as model proteins, demonstrated that the enzymes may be modified with retention of activity. Furthermore, it was found that the properties of the conjugates, organ targeting, blood residence time and immunogenicity are different from the ones obtained with PEG [74] (Table 1).

It is also worth noting that both the polymers were also successfully used to mask the liposome surface for obtaining new forms of “stealth liposomes” (see Table 2) [75]. This result is related to the great flexibility and hydrophilicity of PVP and PACM, requisites that are considered essential to convey stealth properties [76] (Table 3).

Table 1
Influence of PEG molecular mass on the clearance time in rats^a or mice^b. In each case three PEG chains were bound to SOD molecule

Protein ^c	Half-life
SOD	0.08 ^a
SOD-PEG 1900 Da	1.5 ^a
SOD-PEG 5000 Da	11.0 ^a
SOD-PEG 72000 Da	36.0 ^b

^cAn equal number of PEG chains were bound to SOD molecule.

Table 2
Comparison between degree of modification and residual enzymatic activity of uricase and superoxide dismutase after linear PEG. PACM and PVP modification^a

	PEG		PACM		PVP	
	Modification degree %	Activity %	Modification degree %	Activity %	Modification degree %	Activity %
Uricase ^b	44	40	18	24		
SOD	70 ^c	65 ^c			37 ^b	80 ^b

^aThe modification was carried out with a 5 KDa PEG and 6 KDa PACM or PVP.
^bUricase modification was carried out in the presence of uric acid as active site protection (38).
^cData from (65).

Table 3

Half life in mouse circulation of “stealth” liposomes obtained with linear PEG, branched PEG, PAcM and PVP^a

Liposomes ^b		Half life (min)
3 mol%	PEG/PE	80
“	PEG2/PE	140
“	PAcM/PE	40
“	PVP/PE	90
7 mol%	PEG/PE	230
“	PEG2/PE	230
“	PAcM/PE	130
“	PEV/PE	170
Without any polymer		10

^aThe polymer molecular masses were: PEG 5 KDa, PEG2 10 KDa, (2 × 5 KDa), PAcM and PVP 6 KDa.

^bThe polymer content (3 mol % or 7 mol %) is calculated as a fraction of PE (phosphatidylethanolamine) residue.

17. Sources of PEG

Fluka Chemie AG (Germany) is a source of PEGs of different molecular weights but the quality is not so sure. Polymer Laboratories Ltd (England) supplies ultra-pure PEG or methoxy PEG of low polydispersity to use as standards and also, on demand, small quantities of PEG or functionalized PEG for R & D purposes. Shearwater Polymers Inc. (Huntsville Al., USA) is a company specialized on PEG that supplies a great variety of products activated towards any amino acid residues as well as heterofunctional PEGs and PEG conjugates also.

18. Conclusions

The investigations reviewed here demonstrate how rich in research potential is this recent field of research. In fact, the search for new solutions to still unsolved problems, such as organ-specific targeting of conjugates, or the discovering linear or branched polymers with original structure, may still fascinate the chemist. Furthermore the coming of new PEGylation tools such as dendrimer PEG will further develop the biological and therapeutic applications that, began in the 1970s with the pioneering albumin and catalase PEGylation studies by Davis and Abouchowski [77,78], are now expanding to the field of peptide and non-peptide drugs [79], to immunology [80,81], diagnostics and biocatalysis [82,83].

Acknowledgements

The author would like to thank the CNR (Target Project on Biotechnology) project for financial support. Prof. Milton J. Harris is also acknowledged for reading the manuscript.

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Chemistry for peptide and protein PEGylation

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Received 17 December 2001; accepted 22 January 2002

Abstract

Poly(ethylene glycol) (PEG) is a highly investigated polymer for the covalent modification of biological macromolecules and surfaces for many pharmaceutical and biotechnical applications. In the modification of biological macromolecules, peptides and proteins are of extreme importance. Reasons for PEGylation (i.e. the covalent attachment of PEG) of peptides and proteins are numerous and include shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the reticuloendothelial system (RES), and preventing recognition and degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing the renal filtration and altering biodistribution. An important aspect of PEGylation is the incorporation of various PEG functional groups that are used to attach the PEG to the peptide or protein. In this paper, we review PEG chemistry and methods of preparation with a particular focus on new (second-generation) PEG derivatives, reversible conjugation and PEG structures. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: PEGylation; PEG-protein; PEG conjugation; PEG chemistry

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1. Introduction

The use of proteins and peptides as human therapeutics has expanded in recent years due to: (1) discovery of novel peptides and proteins, (2) a better understanding of the mechanism of action *in vivo*, (3) improvements in expression or synthesis of proteins and peptides that closely resemble fully human proteins and peptides, and (4) improvements in formulation or molecule-altering technologies that have the ability to deliver polypeptides *in vivo* with improved pharmacokinetic and pharmacodynamic properties. It was estimated that in the year 2000, as many as 500 biopharmaceutical products were undergoing clinical trials, and the estimated annual growth rates of protein products (glycoproteins, unglycosylated proteins and antibodies) will range from 10 to 35% [1].

Although more biopharmaceuticals are in development than ever before, many of these have problems that are typical of polypeptide therapeutics, including short circulating half-life, immunogenicity, proteolytic degradation, and low solubility. Several strategies have emerged as ways to improve the pharmacokinetic and pharmacodynamic properties of biopharmaceuticals, including: (1) manipulation of amino acid sequence to decrease immunogenicity and proteolytic cleavage, (2) fusion or conjugation to immunoglobulins and serum proteins, such as albumin, (3) incorporation into drug delivery vehicles for protection and slow release, and (4) conjugating to natural or synthetic polymers [2–6].

Those in the biomedical, biotechnical and pharmaceutical communities have become quite familiar with the improved pharmacological and biological properties that are associated with the covalent attachment of poly(ethylene glycol) or PEG to therapeutically useful polypeptides. For instance, PEG conjugation can shield antigenic epitopes of the polypeptide, thus reducing reticuloendothelial (RES) clearance and recognition by the immune system and also reducing degradation by proteolytic enzymes. PEG conjugation also increases the apparent size of the polypeptide, thus reducing renal filtration and altering biodistribution. Contributing factors that affect the foregoing properties are: (1) the number of PEG chains attached to the polypeptide, (2) the molecular weight and structure of PEG chains at-

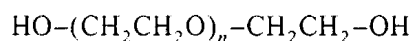
tached to the polypeptide, (3) the location of the PEG sites on the polypeptide and (4) the chemistry used to attach the PEG to the polypeptide.

The importance of chemistry and quality of PEG reagents for peptide and protein modification has only been realized in the last several years as more and more PEG-conjugates have reached late phase clinical trials. The first few PEG-protein products, now on the market (Adagen[®], Oncospar[®], and PEG-Intron[®]), were developed using first generation PEG chemistry. One characteristic of first generation PEG chemistry is the use of low molecular weight linear PEGs (≤ 12 kDa) with chemistry that may result in side reactions or weak linkages upon conjugation with polypeptides.

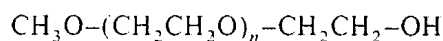
The next generation of PEG-protein therapeutics, which will come to market in the next several years, uses second-generation PEG chemistries. Second-generation PEGylation was designed to avoid the problems of first generation chemistry, notably deactivated PEG impurities, restriction to low molecular weight mPEG, unstable linkages and lack of selectivity in modification. Readers are referred to several detailed reviews on different aspects of PEGylation [7–11]. In this paper, we review chemistries of both first- and second-generation, with an emphasis on newer PEGylation technologies, in order to provide an introduction to those chemistries that will be used in the following reviews.

2. Properties of PEG

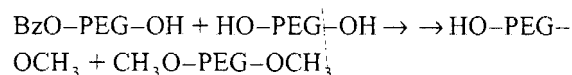
In its most common form poly(ethylene glycol), PEG, is a linear or branched polyether terminated with hydroxyl groups and having the general structure:



PEG is synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. Most useful for polypeptide modification is monomethoxy PEG, mPEG, having the general structure:



Monomethoxy PEG is synthesized by anionic ring opening polymerization initiated with methoxide ions. Commercially available mPEG contains a considerable amount of diol PEG due to the presence of trace amounts of water during polymerization. This diol PEG is also of relatively high molecular weight due to polymerization at both ends of the polymer. The amount of diol PEG can exceed 15% of the composition of mPEG. A solution to the problem of diol contamination has been developed in our laboratories [12]. In this work, a crude benzyloxy-PEG, containing diol impurity, is methylated and then hydrogenated to remove the benzyl group. Thus diol is converted to the inert dimethyl ether, which can be subsequently removed after activation and polypeptide attachment.



Another common route to remove diol is to convert the PEGs to PEG-carboxylic acids that can then be purified by ion-exchange chromatography [13]. PEG with various end groups can be prepared by use of suitable initiator and/or termination reagents. Numerous functionalities can be introduced as end groups on PEG in this manner, including heterobifunctional products. For instance, Kataoka et al. synthesized a heterobifunctional PEG derivative containing aldehyde and thiol end groups [14]. Polymerization was initiated with 3,3-diethoxy-1-propanol, which forms a propionaldehyde after acid hydrolysis, and the polymerization was terminated with methansulfonyl chloride with successive conversion to ethyldithiocarbonate and a free thiol.

Compared with other polymers, PEG has a relatively narrow polydispersity (M_w/M_n) in the range of 1.01 for low molecular weight PEGs (<5 kDa) to 1.1 for high molecular weight PEGs (>50 kDa).

The unique ability of PEG to be soluble in both aqueous solutions and organic solvents makes it suitable for end group derivatization and chemical conjugation to biological molecules under mild physiological conditions. Studies of PEG in solution have shown that PEG typically binds 2–3 water molecules per ethylene oxide unit. Due to both the high flexibility of the backbone chain and the binding of water molecules, the PEG molecule acts

as if it were five to 10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins [15], exclude proteins and cells from surfaces [16], reduce immunogenicity and antigenicity [17] and prevent degradation by mammalian cells and enzymes [18].

Low molecular weight oligomers of PEG (<400 Da) have been shown to be degraded in vivo by alcohol dehydrogenase to toxic metabolites, however the lack of toxicity of PEGs with a molecular weight above 1000 Da has been revealed over many years of use in foods, cosmetics and pharmaceuticals [18].

PEG is rapidly cleared in vivo without structural change and clearance is dependent on molecular weight. Below a molecular weight of about 20 kDa the molecule is cleared in the urine, and higher molecular weight PEGs are cleared more slowly in the urine and feces. PEG is only weakly immunogenic even at high molecular weights. Antibodies to PEG have been generated when attached to a highly immunogenic molecule under an immunization protocol with Freund's adjuvant [19–21]. There are no known situations in which anti-PEG antibodies have been generated under 'normal' clinical administration of a PEG-modified protein.

3. Chemistry of pegylation

To couple PEG to a molecule (i.e. polypeptides, polysaccharides, polynucleotides and small organic molecules) it is necessary to activate the PEG by preparing a derivative of the PEG having a functional group at one or both termini. The functional group is chosen based on the type of available reactive group on the molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group and the C-terminal carboxylic acid. In the case of glycoproteins, vicinal hydroxyl groups can be oxidized with periodate to form two reactive formyl moieties.

The most common route for PEG conjugation of proteins has been to activate the PEG with functional groups suitable for reaction with lysine and N-terminal amino acid groups. Lysine is one of the most

prevalent amino acids in proteins and can be upwards of 10% of the overall amino acid sequence. In reactions between electrophilically activated PEG and nucleophilic amino acids, it is typical that several amines are substituted. When multiple lysines have been modified, a heterogeneous mixture is produced that is composed of a population of several polyethylene glycol molecules attached per protein molecule ('PEGmers') ranging from zero to the number of ϵ - and α -amine groups in the protein. For a protein molecule that has a single PEG attached by this nonspecific modification method, the polyethylene glycol moiety may be attached at a number of different amine sites. Therefore there is the potential for a large number of positional isomers (P) as the degree of modification increases:

$$P = \frac{N!}{(N - k)! \times k!}$$

where N is the number of possible sites and k is the number of sites modified. The extent of modification is important in determining the pharmacological properties of the bioconjugate. Typically, a higher degree of modification will extend the circulation half-life and reduce the likelihood of antigenicity [22]. Each positional isomer of the heterogeneous mixture is likely to have an influence on whether the conjugate is active or whether an antibody will bind an antigenic epitope. The heterogeneity in lysine substitution and in PEG molecular weights is of some concern for PEG-protein pharmaceuticals, and it is generally necessary to demonstrate that the pattern for a particular pharmaceutical can be measured and is reproducible. Many of the important benefits of PEGylation can be controlled by proper conjugation of various molecular weight PEGs to the protein at specific locations on the protein's surface.

The monofunctionality of methoxyPEG makes it particularly suitable for protein and peptide modification because it yields reactive PEGs that do not produce crosslinked polypeptides, as long as diol PEG has been removed. As we will see in the discussion of second generation PEGylation, it is also possible in some instances to reduce or eliminate heterogeneity in the position of substitution.

3.1. First-generation PEG chemistry

3.1.1. PEG chemistry for amine conjugation

Since most applications of PEG conjugation involve labile molecules, the coupling reactions require mild chemical conditions. In the case of polypeptides, the most common reactive groups involved in coupling are the alpha or epsilon amino groups of lysine. In Fig. 1 is listed a wide range of first generation PEG derivatives used for protein PEGylation of either the alpha or epsilon amino groups. First-generation chemistries are generally plagued by PEG impurities, restriction to low molecular weights, unstable linkages, and lack of selectivity in modification. Examples of first-generation PEG derivatives include: (a) PEG dichlorotriazine, (b) PEG tresylate, (c) PEG succinimidyl carbonate, (d) PEG benzotriazole carbonate, (e) PEG *p*-nitrophenyl carbonate, (f) PEG trichlorophenyl carbonate, (g) PEG carbonylimidazole and (h) PEG succinimidyl succinate.

The initial work of Davis et al. used cyanuric chloride to prepare activated PEG for attachment to proteins [6,17]. The PEG dichlorotriazine (Fig. 1a) derivative can react with multiple nucleophilic functional groups such as lysine, serine, tyrosine, cysteine, and histidine, which results in displacement of one of the chlorides and produces a conjugate with retained charge in the form of a secondary amine linkage [23]. The remaining chloride is less susceptible to reactions with nucleophilic residues. Unfortunately, the reactivity is sufficient to allow crosslinking of protein molecules containing additional nucleophilic residues. To solve this problem, Inada et al. synthesized 2,4-bis(methoxypolyethylene glycol)-6-chloro-s-triazine (mPEG₂-chlorotriazine) as shown in Fig. 2 [24]. The lower reactivity of the remaining chlorine translates into a more selective modification of lysine and cysteine residues without further side reactions.

Another alkylating reagent used to nonspecifically modify multiple amino groups to form secondary amine linkages to proteins, viruses and liposomes is PEG tresylate (Fig. 1b) [25]. Although more specific to amino groups than PEG dichlorotriazine, the chemistry of conjugation and the conjugation products are not unique and well defined. For example,

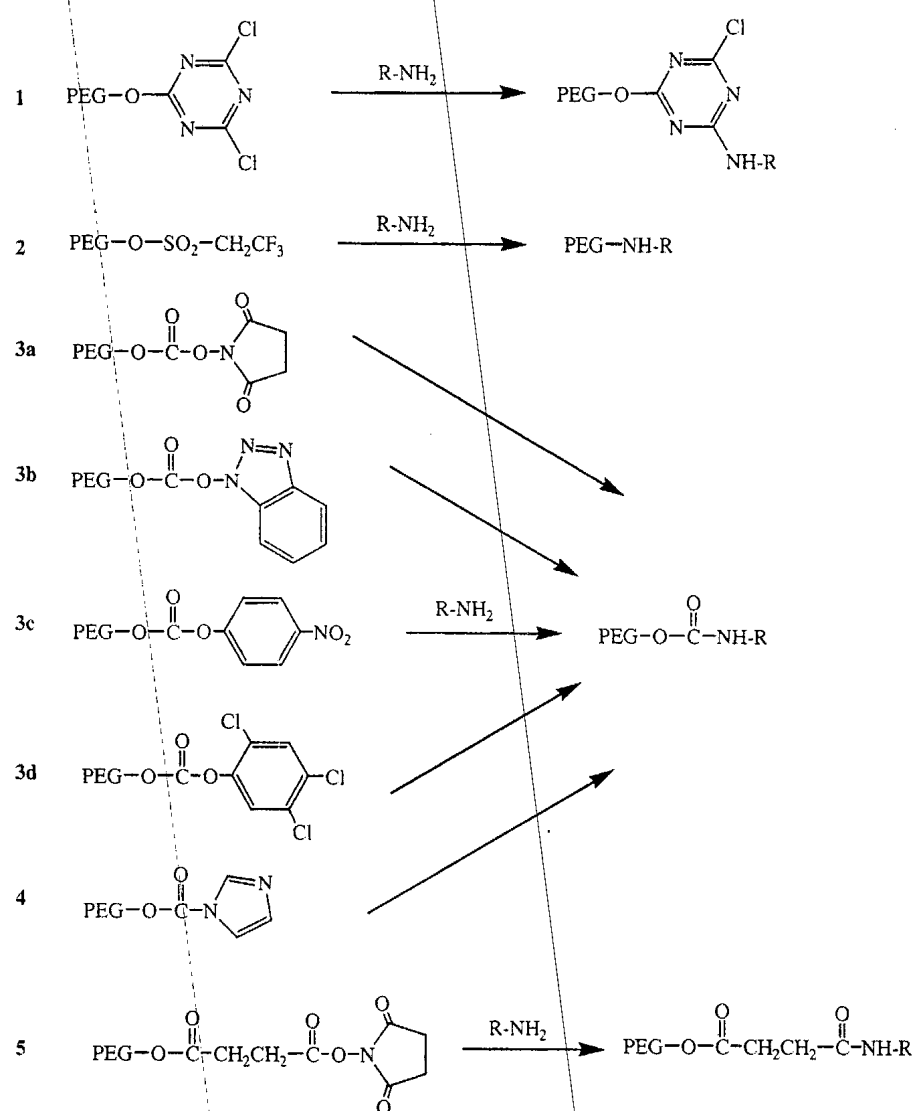


Fig. 1. First-generation amine reactive PEG derivatives.

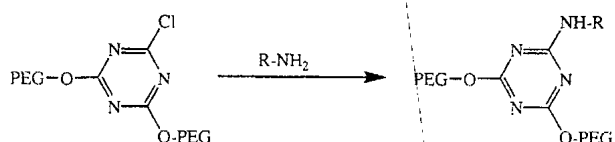


Fig. 2. Branched PEG (PEG2) based on PEG-triazine.

Gais et al. have shown that PEG-tresylate conjugation to small molecule amines can produce a product that contains a degradable sulfamate linkage [26]. Therefore, a heterogeneous mixture that results from

attaching PEG-tresylate to proteins may contain a population of conjugates with degradable linkages.

Most first-generation PEG chemistries are those that produce conjugates through acylation. Two widely used first-generation activated mPEGs are succinimidyl carbonate (SC-PEG in Fig. 1c) [27,28] and benzotriazole carbonate (BTC-PEG in Fig. 1d) [29]. SC-PEG and BTC-PEG react preferentially with lysine residues to form a carbamate linkage, but are also known to react with histidine and tyrosine residues. SC-PEG is slightly more stable to hy-

hydrolysis than BTC-PEG with a half-life of 20.4 min at pH 8 and 25 °C compared to the 13.5 min hydrolysis half-life of BTC-PEG under the same conditions [30]. It has recently been observed that SC-PEG and BTC-PEG couple to histidine residues of α -interferon at slightly acidic conditions to form a hydrolytically unstable imidazolecarbamate linkage [31]. The weak linkage could be used to advantage in preparation of controlled-release formulation, or it could be a disadvantage if conjugate instability were not desired.

Other PEG acylating reagents which produce urethane linked proteins include *p*-nitrophenyl carbonate (pNPC-PEG in Fig. 1e), trichlorophenyl carbonate (TCP-PEG in Fig. 1f) and carbonylimidazole (CDI-PEG in Fig. 1g) [32,33]. These reagents are prepared by reacting chloroformates or carbonylimidazole with the terminal hydroxyl group on mPEG, and these have much lower reactivity than either the SC-PEG or BTC-PEG. Generally, the slower the reaction the more specific the reagent is to certain amino acid groups of the protein. In this way, some selectivity is achieved. The extent and rate of modification can easily be followed in the case of pNPC-PEG and TCP-PEG by monitoring the phenolate-ion leaving-group by colorimetric analysis.

The remaining first-generation PEG reagent is succinimidyl succinate (SS-PEG in Fig. 1h) [34]. SS-PEG is prepared by reaction of mPEG with succinic anhydride, followed by activation of the carboxylic acid to the succinimidyl ester. The polymer backbone contains a second ester linkage that remains after the conjugation reaction with a protein. This linkage is highly susceptible to hydrolysis after the polymer has been attached to the protein. Not only does this hydrolysis lead to loss of the benefits of PEG attachment, but the succinate tag that remains on the protein after hydrolysis can act as a hapten and lead to immunogenicity of the remaining protein [35].

Techniques used to form first generation PEG derivatives are generally straightforward and involve reacting the PEG polymer with a group that is reactive with hydroxyl groups, typically anhydrides, chlorides, chloroformates and carbonates. With the exception of the work by Bentley et al., these techniques lack the ability to produce pure mono-functional PEG derivatives of high molecular weight

[12]. Since the diol content of high molecular weight PEGs can reach 15%, high-molecular-weight, first-generation PEG chemistry is inefficient for protein conjugation. The ability to generate an intermediate that can be purified from deactivated species renders second-generation chemistry a valuable tool for protein modification.

3.2. Second-generation PEGylation chemistry

3.2.1. PEG chemistry for amine conjugation

Second-generation PEGylation chemistry has been designed to avoid the above noted problems of diol contamination, restriction to low molecular weight mPEG, unstable linkages, side reactions and lack of selectivity in substitution. One of the first examples of second-generation chemistry is mPEG-propionaldehyde [36]. mPEG-propionaldehyde is easier to prepare and use than PEG-acetaldehyde because the acetaldehyde is very susceptible to dimerization via aldol condensation. A key property of mPEG-propionaldehyde, as disclosed by Kinstler et al. in work on PEGylation of G-CSF, sTNF-RI, and consensus IFN, is that under acidic conditions (approximately pH 5), aldehyde is largely selective for the N-terminal α -amine because of the lower pK_a of the α -amine compared to other nucleophiles [37–39]. The conjugation of electrophilic PEGs to amino acid residues on proteins is highly dependent on the nucleophilicity of each amino acid residue. Nucleophilic attack will only take place when the pH of the protein solution is near or above the residue's pK_a . Therefore the reactivity of each residue also depends on neighboring amino acid residues. Although complete selectivity is not observed, the extensive heterogeneity frequently seen with lysine chemistry is greatly reduced. Coupling of aldehydes to primary amines proceeds through a Schiff base, which is reduced in situ to give a stable secondary amine linkage as shown in Fig. 3.

An alternative approach to using PEG-aldehyde is to use the acetal derivative of PEG-propionaldehyde or PEG-acetaldehyde [40]. The aldehyde hydrate of the acetal derivatives can be generated in situ by acid hydrolysis (Fig. 4). The pH of the solution can then be adjusted to values sufficient for protein modification with the same mechanism as the free aldehyde derivative in Fig. 3. The benefit of using the

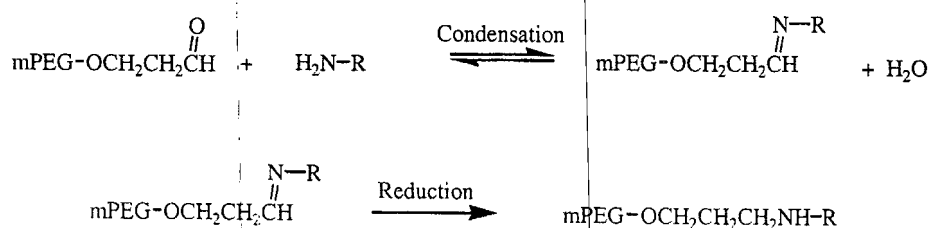


Fig. 3. Reductive amination using PEG-propionaldehyde.

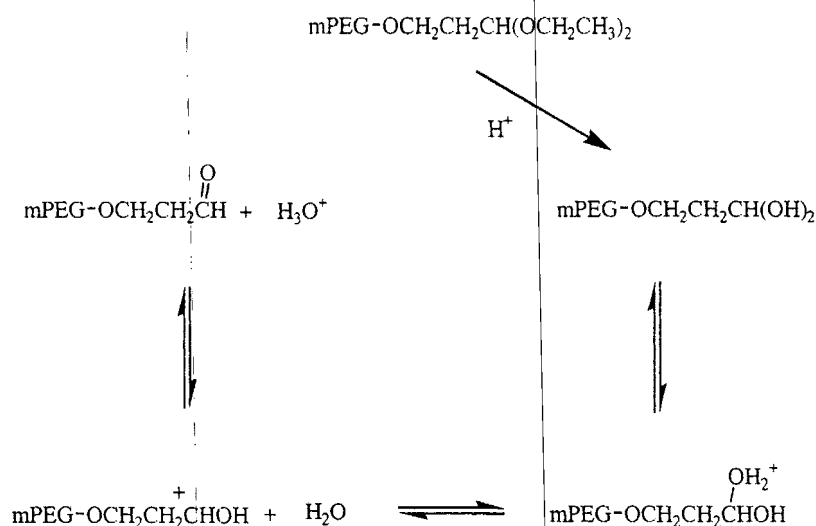


Fig. 4. In situ generation of PEG-aldehyde hydrate for use in reductive amination.

acetal derivative over the free propionaldehyde or acetaldehyde is longer storage stability and higher purity.

Active esters of PEG carboxylic acids are the most used acylating agents for protein modification. Active esters react with primary amines near physiological conditions to form stable amides as shown in Fig. 5. Generating the carboxylic acid intermediate allows the PEG to be purified from unsubstituted or disubstituted impurities by ion-exchange chromatography [41]. Purities of greater than 97% are routinely obtainable by this method. Activation of PEG-carboxylic acids to the succinimidyl active esters is accomplished by reacting the PEG-carboxylic acid with *N*-hydroxysuccinimide (NHS or HOSu) and a carbodiimide.

The first carboxylic acid derivative of PEG not containing a degradable linkage to the PEG backbone, as in SS-PEG, was carboxymethylated PEG

(CM-PEG) [42]. The succinimidyl ester of this compound (SCM-PEG) is extremely reactive (hydrolysis $t_{1/2}$ of 0.75 min at pH 8 and 25 °C) and is therefore difficult to use. To take advantage of the intermediate purification step and have an active ester that had more favorable kinetics for protein modification, Harris et al. prepared propionic acid (PEG-O-CH₂CH₂-COOH) and butanoic acid (PEG-O-CH₂CH₂CH₂-COOH) derivatives of PEG (Fig. 5(1)) [13]. Changing the distance between the active ester and the PEG backbone by the addition of methylene units had a profound influence on the reactivity towards amines and water. For example, SBA-PEG, which has two additional methylene groups, has a longer hydrolysis half-life of 23 min at pH 8 and 25 °C. SPA-PEG, which has one additional methylene group, has a hydrolysis half-life of 16.5 min at pH 8 and 25 °C.

Reactivity of PEG active esters towards amines

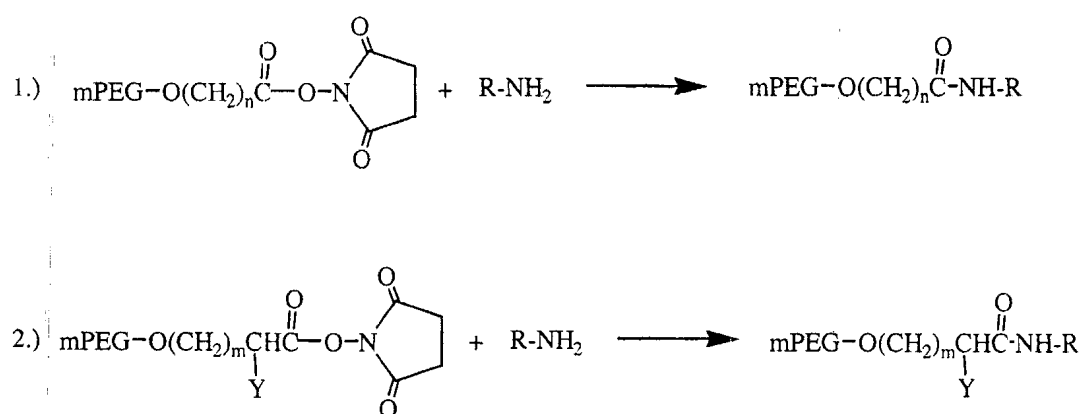


Fig. 5. PEG NHS esters. (1) PEG NHS esters based on propionic and butanoic acids and (2) α -branched PEG NHS esters based on propionic and butanoic acids.

and water can be further decreased by introducing an α -branching moiety to the carboxylic acid as shown in Fig. 5(2). An α -methyl branched PA-PEG derivative has a hydrolysis half-life of 33 min at pH 8 and 25 °C.

3.2.2. PEG chemistry for cysteine modification

PEGylation of free cysteine residues in proteins is the main approach for site-specific modification because reagents that specifically react with cysteines have been synthesized, and the number of free cysteines on the surface of a protein is much less than that of lysine residues. In the absence of a free cysteine in a native protein, one or more free cysteines can be added by genetic engineering [43]. The advantage of this approach is that it makes possible site-specific PEGylation at areas on the protein that will minimize a loss in biological activity but decrease immunogenicity. This strategy is not without its shortcomings. The addition of free cysteines by genetic engineering increases the possibility of incorrect disulfide formation and protein dimerization.

PEG derivatives such as PEG-maleimide (Fig. 6(1)), vinylsulfone (Fig. 6(2)), iodoacetamide (Fig. 6(3)), and orthopyridyl disulfide (Fig. 6(4)) have been developed for PEGylation of cysteine residues, with each derivative having its own advantages and disadvantages [43–46]. PEG-vinylsulfone (PEG-VS) reacts slowly with thiols to form a stable thioether linkage to the protein at slightly basic conditions (pH 7–8) but will proceed faster if the pH is increased.

Although PEG-VS is stable in aqueous solutions, it may react with lysine residues at elevated pH. Unlike PEG-VS, PEG-maleimide (PEG-MAL) is more reactive to thiols even under acidic conditions (pH 6–7), but it is not stable in water and can undergo ring opening or addition of water across the double bond. PEG-iodoacetamide (PEG-IA) reacts slowly with free thiols by nucleophilic substitution, creating a stable thioether linkage. The reaction should be done in slight molar excess of PEG-IA in a dark container to limit the generation of free iodine that may react with other amino acids. The thioether linkage between the PEG-MAL and protein is stable, but slow cleavage of one of the amide linkages can occur by hydrolysis. Orthopyridyl disulfide-PEG (PEG-OPSS) reacts specifically with sulfhydryl groups under both acidic and basic conditions (pH 3–10) to form a disulfide bond with the protein. Disulfide linkages are also stable, except in a reducing environment when the linkage is converted to thiols.

Scientists in our laboratories recently prepared a highly active, long circulating and stable conjugate of IFN- β using a two-step method with PEG-OPSS [47]. The tertiary structure of IFN- β was determined by Karpusas et al. who showed that the free cysteine residue at position 17 was proximal to the surface but hidden [48]. In this case, the available thiol was not accessible to high molecular weight PEG that would be needed for improved pharmacokinetics. The approach that was ultimately adopted was to couple a low molecular weight di-OPSS PEG (M_w 2000) to the interferon and then couple a PEG thiol

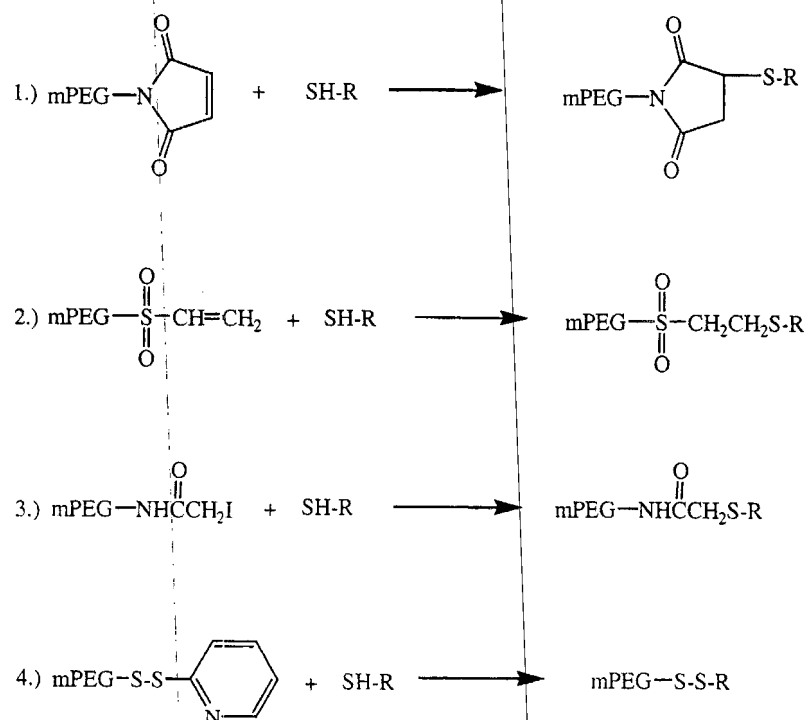


Fig. 6. Thiol reactive PEGs. (1) PEG maleimide, (2) PEG vinyl sulfone, (3) PEG iodoacetamide, and (4) PEG orthopyridyl disulfide.

to the remaining terminal OPSS group. The disulfide linkage between PEG and the protein was found to be stable in plasma circulation (unpublished data).

3.2.3. PEG chemistry for oxidized carbohydrates or N-terminus

Oxidation of carbohydrate residues or N-terminal serine or threonine is an alternative method for site-directed PEGylation of proteins. Carbohydrates can be oxidized with enzymes, such as glucose oxidase, or chemically with sodium periodate. Oxidation of the carbohydrate residues generates multiple reactive aldehyde groups, which can be reacted with either PEG-hydrazide to produce a hydrazone linkage or with PEG-amine to produce a reversible Schiff base (Fig. 7) [49]. The hydrazone linkage may be reduced with sodium cyanoborohydride to a more stable alkyl hydrazide and the Schiff's base may be reduced to form a secondary amine. Reductive alkylation with PEG-amine is problematic because the amino groups of a protein possess similar reactivity to PEG-amines and thus may form cross-

linked aggregates. PEG-hydrazides are more useful in these situations. Under acidic conditions (approx. pH 5), amino groups of the protein are predominantly protonated, but because the PEG-hydrazide is a weaker base (pK_a approx. 3) than primary amines (pK_a approx. 10), the reaction is selective to the PEG-hydrazone formation. Multiple attachment sites are generated using this method, but the modification site is specific to the carbohydrate.

Another approach to site-specific conjugation is to take advantage of the presence of a N-terminal serine or threonine, which can be converted by periodate oxidation to a glyoxylyl derivative. Gaertner et al. oxidized the N-terminal serine of IL-8 to form a glyoxylyl derivative, which they conjugated to aminooxy and hydrazide PEG derivatives [50].

3.2.4. PEG chemistry for reversible PEGylation

Most PEGylation chemistry is designed to create a conjugate that contains a stable linkage to the protein. In most cases having a stable linkage to the protein is beneficial because of the suitability for

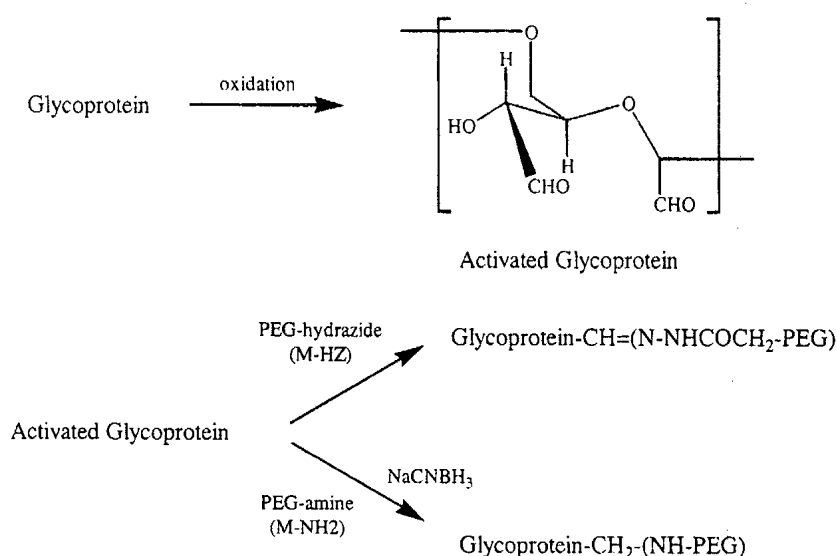


Fig. 7. Attachment of poly(ethylene glycol) to oxidized carbohydrates of glycoproteins.

long-term storage, easier purification and availability of prefilled syringes. It is also generally observed that stable linkages to a protein can reduce the activity, possibly due to the presence of the PEG chain at the active or binding site of the protein or steric crowding at the active or binding site. Also the PEG molecular weight has a direct impact on the activity; higher molecular weight PEG conjugates tend to have lower in vitro activity but have higher in vivo activity due to the improved pharmacokinetic profile [51]. The objective of most PEG conjugation techniques is to increase the circulation half-life without altering activity. In the development of PEG-Intron[®], Enzon used a degradable linkage between the PEG and protein to improve the pharmacokinetic half-life but minimize loss of activity by releasing native interferon alpha-2b [52]. PEG-Intron is formed by conjugation of PEG-SC to interferon alpha-2b at low pH (around pH 5). The conjugation leads to a population of PEG conjugates coupled to His³⁴ (Fig. 8). In this case, the PEG is coupled to the N^{δ1} position of the imidazole ring in histidine to form a carbamate linkage and the PEG was found to be released from the protein over time. Note should be taken when comparing PEG-Intron to the branched PEG_{40 kDa}-interferon alpha-2a conjugate (Pegasys[®]) that the PEG-Intron product has a higher in vitro activity compared to Pegasys, but the in vivo

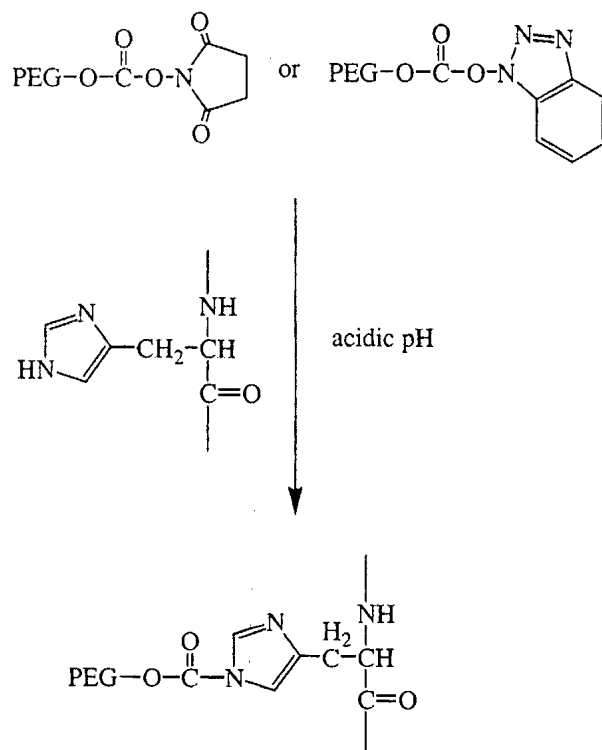


Fig. 8. Attachment of PEG-benzotriazole or PEG-succinimidyl carbonate to histidine residues on proteins.

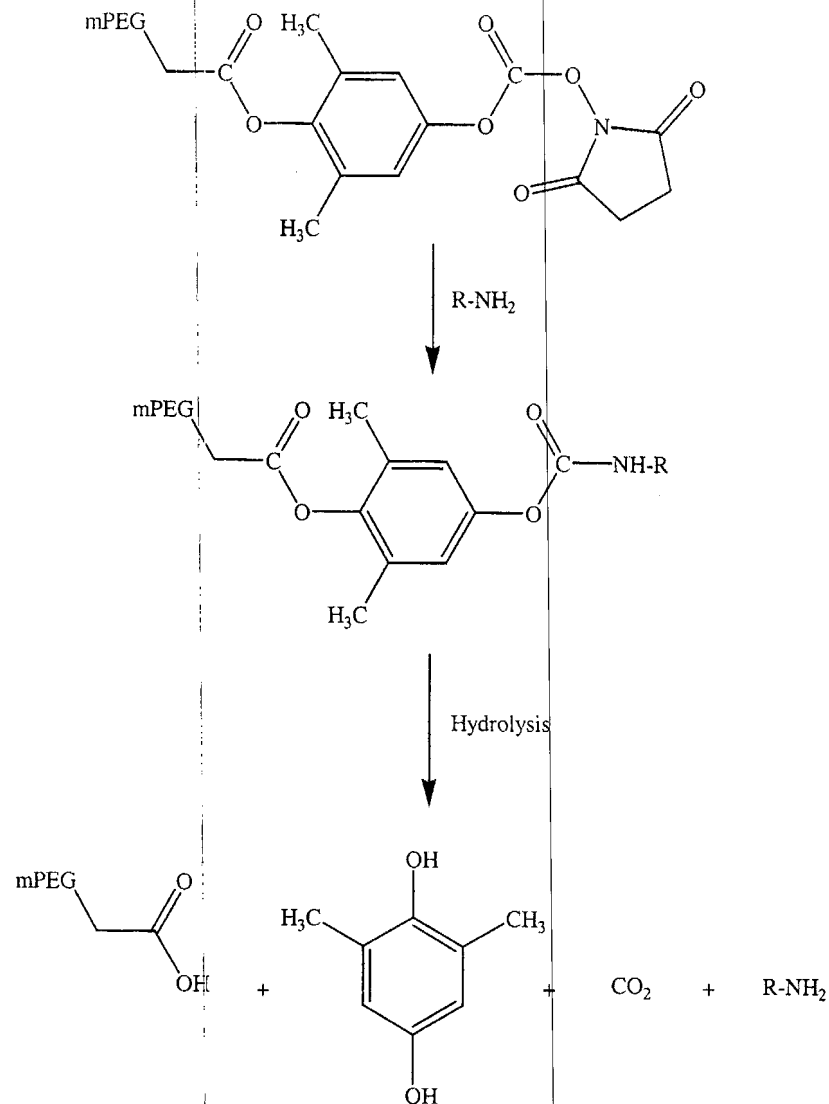


Fig. 12. Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (1,6-elimination mechanism).

is to simply limit molar equivalents of each reagent. However, a distribution of substitution will result as shown below.

HO-PEG-OH

HO-PEG-COOH

HOOC-PEG-COOH

A chromatographic approach was used by Zalipsky

et al. to purify a PEG acid that was synthesized by reacting glycine with a PEG succinimidyl carbonate derivative [40]. Ion exchange chromatography was shown to be a very effective purification method to separate monoacid from diacid and unsubstituted PEG.

Bentley et al. prepared heterobifunctional PEGs by first initiating PEG polymerization with benzyl alcohol to produce benzyl PEG (Bz-PEG-OH) [60]. The hydroxyl group of the intermediate polymer is

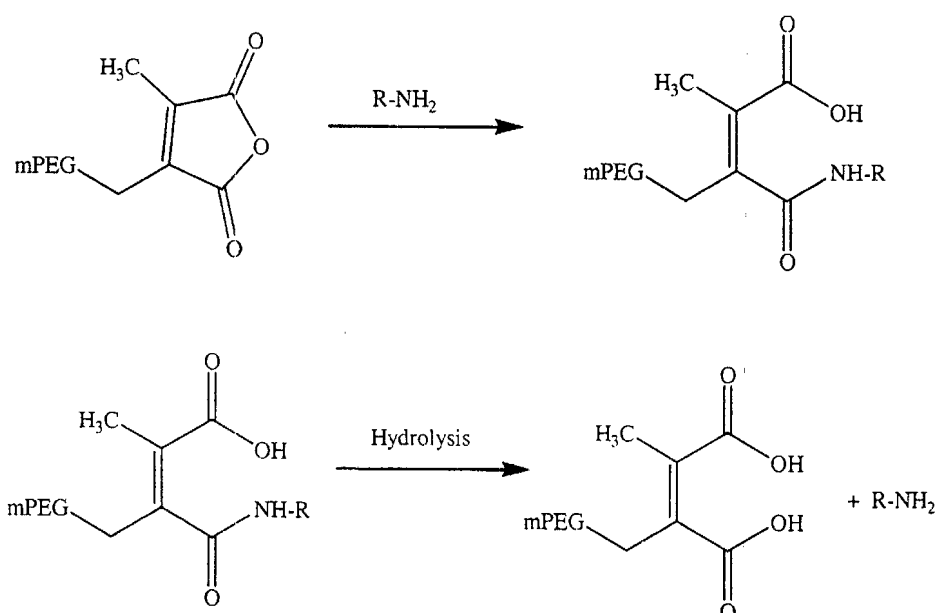


Fig. 10. Attachment and release of PEG-methylmaleic anhydride from amine containing drugs.

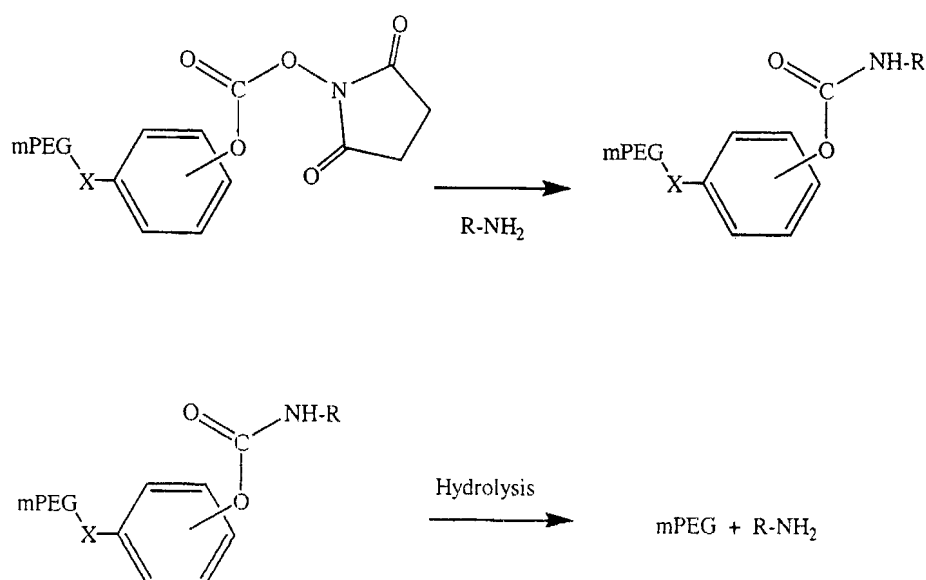


Fig. 11. Attachment and release of PEG-phenyl NHS carbonates from amine containing drugs (hydrolysis mechanism) [X is O or $-CONH-$].

Several methods have been developed to synthesize heterobifunctional PEGs, each having its own advantages and disadvantages. Preferred end groups

for heterobifunctional PEGs are NHS esters, maleimide, vinyl sulfone, pyridyl disulfide, amine, and carboxylic acids. The first such synthetic method

activity of the Pegasys product is higher due to the superior pharmacokinetic profile [53,54].

An approach to regaining protein activity lost by PEGylation is the use of PEG chemistry that releases the native protein over time through enzymatic degradation, hydrolytic cleavage or reduction. The first such PEG reagent was PEG-succinimidyl succinate, described above. Other 'double ester' PEG reagents have been investigated by Roberts et al. to help control the release rates of the protein and regain activity over a period of time [55]. In this case, hydroxy acids are attached to carboxylic acids of PEG (carboxymethyl, propionic, or butanoic) to create a PEG acid that has an ester linkage between the hydroxy acid and PEG acid (Fig. 9). The terminal acid of the PEG derivative can then be activated and attached to α - and ε -amino groups of proteins. Regeneration of at least 60% of the native activity of lysozyme was recovered at physiological conditions of completely inactivated protein after release of the PEG from the protein. The problem with the double ester PEG reagents is that they release a protein that contains a 'tag' that could lead to immunogenicity of the protein.

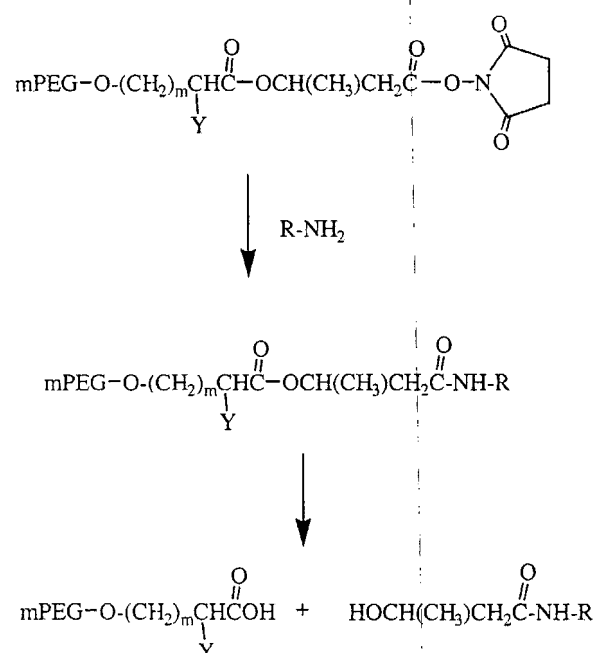


Fig. 9. Attachment and release of PEG-double esters from proteins (Y is an aliphatic or aromatic moiety).

To circumvent the loss of activity associated with some PEG modifications and the potential immunogenic nature of proteins released from PEG with a 'tag' as described above, reagents that release the native protein without 'tags' seem to be a better choice for protein modification. The first such reagent was PEG maleic anhydride used by Garman et al. to attach PEG to tissue plasminogen activator and urokinase (Fig. 10) [56]. Both of the conjugates regenerated the native protein under physiological conditions and had a 5–10 $\times$ slower clearance rate than the native protein in the guinea pig.

Another example of a releasable PEG reagent was prepared by Bentley et al. [57]. In this work, mPEG phenyl ether succinimidyl carbonates and mPEG benzamide succinimidyl carbonates (Fig. 11) are used to conjugate to amino groups on lysozyme. Both conjugates regenerated the native protein under physiological conditions and the rate of release was controlled by the substitution position on the phenyl.

Greenwald et al. also synthesized a releasable PEG reagent that released native protein by a 1,6 elimination mechanism (Fig. 12) [58]. Again, the native protein was regenerated with nearly 100% of its bioactivity.

A further example of a releasable PEG was proposed by Zalipsky et al., which released the native protein by a mechanism other than hydrolysis [59]. The linkage as shown in Fig. 13 employs a *p*- or *o*-disulfide of a benzyl urethane. When subjected to mild reducing environments, such as that present in endosomal compartments of a cell, the original amine component is regenerated.

3.2.5. Heterobifunctional PEG chemistry

As applications of PEG chemistry have become more sophisticated, there has been an increasing need for heterobifunctional PEGs, which are PEGs bearing dissimilar terminal groups. Such heterobifunctional PEGs bearing appropriate functional groups may be used to link two entities where a hydrophilic, flexible, and biocompatible spacer is needed. Heterobifunctional PEG can be used in a variety of ways that includes linking macromolecules to surfaces (for immunoassays, biosensors or various probe applications), targeting of drugs, liposomes and viruses to specific tissues, liquid phase peptide synthesis and many others.

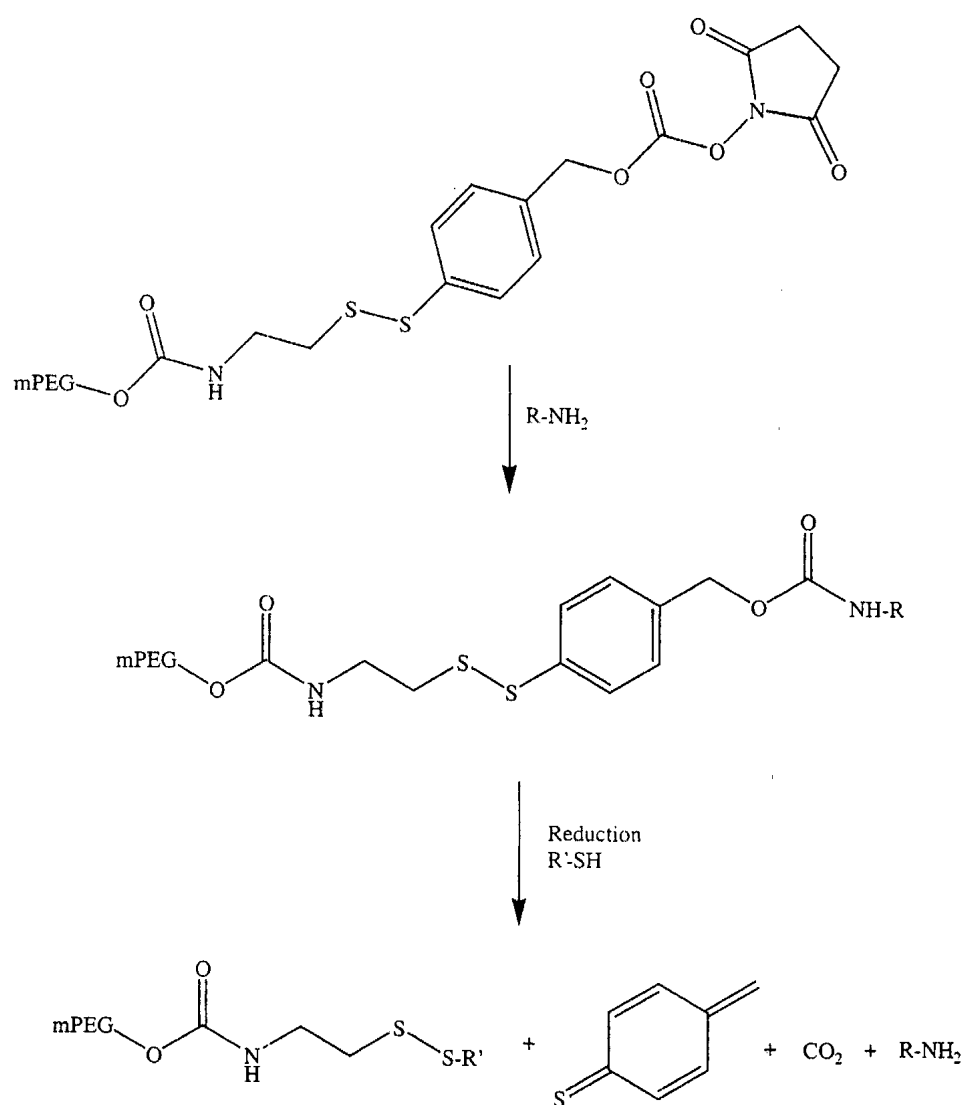


Fig. 13. Attachment and release of PEG phenyl NHS carbonates from amine containing drugs (reduction mechanism).

converted to a first reactive functional group. The benzyl group can then be removed by hydrogenolysis or hydrolysis, without chemically affecting the first functional group, thus making available a new terminal hydroxyl group for the addition of a second functional group.

A promising strategy for generating heterobifunctional PEGs is the polymerization approach. Initiating polymerization of ethylene oxide with an anion that ultimately becomes the end-group of PEG is the most direct route for heterobifunctional synthesis. Terminating the reaction with water generates a

hydroxyl group at the second terminal end that can undergo activation by chemical methods. For example, this method has been used by Yokoyama et al. to prepare a PEG with a hydroxyl group at one terminus and an amino group at the other [61]. Cammas et al. have also used this method to prepare PEGs with an amino group on one terminus and a hydroxyl or methoxy group on the other [62]. It has also been used by Nagasaki et al. to prepare a PEG having a formyl group at one terminus and a hydroxyl group at the other [63]. This strategy also has its limits. Only those anions that are desirable as

end groups and suitable for initiating polymerization are useful for synthesis of heterobifunctional PEG by this route. This method is also limited by the fact that rigorous exclusion of water is necessary to prevent the formation of diol. This problem becomes more severe as PEG molecular weight increases. If these limits are resolved, generating heterobifunctional PEG reagents by anionic polymerization may be the most efficient and cost effective method.

3.3. PEG structures

In addition to the linear structure of the PEG molecule shown above, branched structures have proven useful for protein and peptide modification. The first branched PEG structure, 2,4-bis(methoxypolyethylene glycol)-6-chloro-s-triazine (mPEG₂-chlorotriazine), was based on a triazine core and synthesized by Inada et al. as shown in Fig. 2 [24].

Yamasaki et al. first synthesized a more useful branched PEG structure, based on a lysine core [64]. A highly purified branched PEG or PEG2 (Fig. 14A) was constructed by Veronese et al. using two linear PEG-BTC (or the related PEG-SC) chains linked to

the α - and ϵ -amino groups of lysine [65]. This construct allows for a large molecular weight (upward of 60 kDa) and highly pure PEG to be synthesized with a single reactive end group. The standard preparation of PEG2 intermediate acid contains impurities. These impurities consist of unreacted PEG-BTC, a lysine residue that has only one PEG chain attached to one of the amino groups ('PEG1'), and 'PEG3', which is a linear PEG having a lysine at each end of a diactivated PEG impurity and two mPEG-BTC molecules coupled to the remaining two amino groups (thus PEG3 is a diacid). During aqueous work-up, the unreacted mPEG-BTC is converted back to mPEG-OH. Thus the reaction mixture contains a diacid, a zwitterion, a neutral mPEG, and the desired monoacid, which can be purified by careful ion-exchange chromatography. The acid of the lysine linker can be converted into a range of other derivatives including NHS esters, aldehydes, thiols and maleimide.

PEG2 turns out to be a very exciting protein PEGylation reagent because of its unique characteristics when compared to linear PEGs. For example, PEG2 attached to proteins 'acts' much larger than a corresponding linear mPEG of the same M_w [64]. This structure of PEG also has the advantage of adding two PEG chains per attachment site on the protein, therefore reducing the chance of protein inactivation. Furthermore, the PEG2 structure is more effective in protecting proteins from proteolysis, reducing antigenicity and reducing immunogenicity [66].

Another PEG is the forked PEG. Instead of having a single functional group at the end of two PEG chains, as with PEG2, forked PEG has two reactive groups at one end of a single PEG chain or branched PEG (Fig. 14B,C). Harris et al. first synthesized a forked PEG by attaching to the terminus of a polymer backbone a single functional group of a trifunctional linker, such as serinol or β -glutamic acid [67]. The remaining proximal functional groups, which are linked to a central carbon atom, are able to react with two other molecules, which can be the same or different, to produce a PEG molecule that contains two molecules at a single terminus of the PEG chain.

Arnold et al. synthesized PEG compounds having terminal metal chelating groups, which consisted of

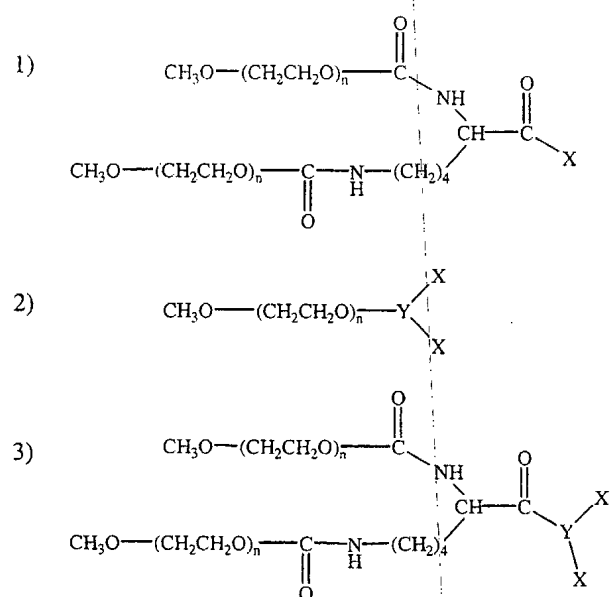


Fig. 14. Various PEG structures. (1) Branched PEG (PEG2), (2) linear forked PEG, and (3) branched forked PEG [Y is a group having a carbon branching moiety and X is a molecule, linker and/or a functional group].

two free carboxylic acid or amino groups linked to a central nitrogen atom [68]. The PEG compounds were used to extract and precipitate proteins from solutions with the carboxylic acid or amino groups together with the nitrogen atom capable of forming ionic complexes with metal ions. Similar PEG compounds were synthesized by Martinez et al. to link hydroxyl containing moieties to the two terminal carboxylic acid groups to create a PEG-linked pro-drug [69].

Forked PEG is useful for conjugating molecules where the benefit would be to bring two moieties in close proximity to one another, for example, dimerization of cell surface receptors to activate cellular mechanisms. Also, of interest is conjugating a F(ab)' fragment of an antibody to the proximal reactive groups to produce a conjugate that closely resembles the structure of the full-length antibody. Another useful attribute of forked PEG is the increased loading capacity of small molecule pharmaceuticals.

4. Conclusions

The array of PEG chemistries reviewed here are among dozens being used for clinical development of PEGylated peptides and proteins. The transition from first-generation chemistries to second-generation chemistries is taking place at a rapid pace and future demands for PEG reagents will lead to new reagents for novel applications in the biopharmaceutical industry. Novel PEG chemistry for site-specific modification, as well as control of PK/PD parameters, will be synthesized when the needs arise. The importance of chemistry and quality of PEG reagents for peptide and protein modification has only been realized in the last several years as more and more PEG-conjugates make it to late phase clinical trials. Clearly, the scientific community is eagerly awaiting the results of the ongoing clinical trials to determine future product candidates. We expect that the approval of PEG-Intron for Hepatitis C and the FDA filing of Pegasys (PEG-IFN- α 2a), PEG-Neupogen[®] (PEG-G-CSF) and PEGVisomant (PEG-hGHra) will bring new life to a seasoned technology. What was thought to be a failing technology is now fulfilling its long recognized potential.

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Functional Mapping of Receptor Specificity Domains of Glial Cell Line-derived Neurotrophic Factor (GDNF) Family Ligands and Production of GFR α 1 RET-specific Agonists*

(Received for publication, September 17, 1999, and in revised form, November 12, 1999)

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The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) (GDNF, neurturin, artemin, and persephin) are critical regulators of neurodevelopment and support the survival of midbrain dopaminergic and spinal motor neurons *in vitro* and in animal disease models making them attractive therapeutic candidates for treatment of neurodegenerative diseases. The GFLs signal through a multicomponent receptor complex comprised of a high affinity binding component (GDNF-family receptor α -component (GFR α 1-GFR α 4)) and the receptor tyrosine kinase RET. To begin characterization of GFL receptor specificity at the molecular level, we performed comprehensive homologue-scanning mutagenesis of GDNF, the prototypical member of the GFLs. Replacing short segments of GDNF with the homologous segments from persephin (PSPN) (which cannot bind or activate GFR α 1-RET or GFR α 2-RET) identified sites along the second finger of GDNF critical for activating the GFR α 1-RET and GFR α 2-RET receptor complexes. Furthermore, introduction of these regions from GDNF, neurturin, or artemin into PSPN demonstrated that they are sufficient for activating GFR α 1-RET, but additional determinants are required for interaction with the other GFR α s. This difference in the molecular basis of GFL-GFR α specificity allowed the production of GFR α 1-RET-specific agonists and provides a foundation for understanding of GFL-GFR α -RET signaling at the molecular level.

The glial cell line-derived neurotrophic factor (GDNF)¹ family ligands (GFLs) are distant members of the TGF- β superfamily that are potent neurotrophic factors *in vitro* and are critical for the development of distinct neuronal populations *in vivo* (1). There are currently four known GFLs, GDNF (2), neurturin (NRTN (3)), artemin (ARTN (4)), and persephin (PSPN (5)). Each GFL is a demonstrated survival factor for dopaminergic ventral midbrain neurons cultured from the embryo (2, 4–6). Furthermore, GDNF, NRTN, and PSPN have

also been shown to support the survival of motor neurons in culture (5, 7–9). Therefore there is considerable interest in the therapeutic potential of these molecules for use in treating neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis, which is underscored by the success of GDNF in relieving disease symptoms in several animal models of Parkinson's disease (10). However the difficulty of delivering these large polypeptides to their intended site of action in the central nervous system makes it desirable to understand the molecular basis of their action to aid in attempts to produce minimal agonists of the GFL receptor system.

The GFLs signal through a unique multicomponent receptor complex, consisting of a high affinity glycosylphosphatidylinositol-anchored binding component (GFR α 1-GFR α 4) and the receptor tyrosine kinase RET. Extensive *in vitro* and *in vivo* experimentation has supported the hypothesis that for each GFL there is a preferred GFR α receptor, to which the GFL binds with highest affinity and most potently activates RET. These preferred interactions are GDNF-GFR α 1, NRTN-GFR α 2, and ARTN-GFR α 3 (4, 9, 11, 12). PSPN does not bind or activate any of the mammalian GFR α s, but does bind to a protein currently only identified in chicken called GFR α 4 (5, 13, 14). However, despite these preferential interactions, there is also clear cross-talk between the different ligand-receptor pairs. The known alternative interactions are NRTN-GFR α 1, ARTN-GFR α 1, and GDNF-GFR α 2 (4, 11, 15, 16). As GFR α 1 and GFR α 2 are often expressed by the same or closely neighboring neurons (6, 17), it is still unclear through which receptor exogenously administered GDNF and NRTN mediate their neurotrophic actions on various neuronal populations.

Whereas the *in vitro* interactions between the different GFLs and GFR α s are now relatively well understood, the molecular basis of this specificity and cross-talk is unknown (18). Furthermore, the precise details of receptor complex formation and stoichiometry are also poorly characterized. The crystal structure of GDNF indicates that it is a disulfide-bonded dimer that is significantly similar to the structure of TGF- β 2, as predicted by the cysteine spacing of its primary sequence (19–21). However, the structure itself yields only speculative information regarding receptor binding surfaces. Furthermore, analogy to other TGF- β superfamily members regarding receptor binding surfaces would likely be unfounded as the receptors used by GDNF and the TGF- β s are drastically different and likely hold no structural similarity.

To characterize the structural requirements of GDNF for interacting with its receptors, we performed homologue-scanning mutagenesis, replacing short blocks (~8 amino acids) of GDNF sequence with the homologous blocks from PSPN, which cannot bind or activate GFR α 1-RET or GFR α 2-RET. This screen identified two critical regions for the GDNF-GFR α 1

* This work was supported by National Institutes of Health Grants R01 AG13729 and R01 AG13730 and Genentech. Washington University, E. M. J., and J. M. may receive income based on a license to Genentech. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; NRTN, neurturin; PSPN, persephin; ARTN, artemin; GFL, GDNF family ligand; GFR α , GDNF family receptor α -component; TGF- β , transforming growth factor- β .

interaction and at least one additional region critical for the alternate GDNF-GFR α 2 interaction. These blocks are discontinuous along the primary sequence of GDNF but are directly adjacent along the second finger of GDNF when mapped to the crystal structure. We further demonstrate that these two regions from any of the GFR α 1 agonists (GDNF, NRTN, or ARTN) are sufficient to activate GFR α 1-RET in the context of the PSPN molecule and that these chimeric mutants function as GFR α 1-RET-specific agonists *in vitro*. Finally, we identify an additional region that is critical for the NRTN-GFR α 2 and ARTN-GFR α 3 interactions. These data identify a putative receptor interaction site shared by the GFLs to activate GFR α 1-RET and identify additional determinants required for GDNF and NRTN to activate GFR α 2-RET and for ARTN to activate GFR α 3-RET.

EXPERIMENTAL PROCEDURES

Mutagenesis, Synthesis, and Purification of GFL Mutants—All mutants were produced by fusion polymerase chain reaction mutagenesis. The amino acid sequence of rat GDNF, mouse NRTN and PSPN, and human ARTN was used for all constructs. Polymerase chain reaction products were directly cloned into the *Mlu*I and *Xba*I sites of plasmid pCB6 (22), and the inserts were sequenced entirely. For mutant G-hf-GDNF the 6His-FLAG tag was inserted between the third and fourth residue of mature GDNF, making the sequence (N-... RLKR SPD-HHHHHHDKDDDD-KQAL...-C). For mutant N-hf-GDNF, residues 1–38 were truncated from mature GDNF and attached to the prepro-region of NRTN. The resulting sequence was (N-... RRAR PGA-HHHHHHDKDDDDK-RGCV...-C). For N-hf-ARTN, residues 1–13 were truncated from mature human ARTN and attached to the prepro-region of NRTN, with the resulting sequence (N-... RRAR PGA-HHHHHHDKDDDDK-RGCR...-C). For mutant P-hf-PSPN, the tag was inserted between the third and fourth residue of mature PSPN, resulting in N-... RLPR ALA-HHHHHHDKDDDDK-GSCR...-C. Expression plasmids for homologue-scanning mutants were produced as above by fusion polymerase chain reaction, and all were based on the N-hf-GDNF construct.

For mutant protein production in COS cells, expression plasmids were transfected using the DEAE-dextran/Chloroquine method (23). COS cells were plated onto 10- or 15-cm dishes, transfected, and switched to Dulbecco's modified Eagle's medium containing 1% fetal calf serum. After 4–5 days, conditioned medium was collected, cleared, and either concentrated using Centrprep-10 concentrators (Amicon) or purified by nickel chromatography (Qiagen). Proteins were visualized by immunoblotting with anti-FLAG M2 monoclonal antibody (Sigma). Relative quantities of FLAG-tagged proteins were determined by an enzyme-linked immunosorbent assay also using the anti-FLAG M2 antibody. Briefly, purified proteins or conditioned medium were coated to Nunc-Immuno MaxiSorp microtiter plates overnight at 4 °C. Plates were washed (3 $\times$ in Tris-buffered saline, 0.03% Tween 20), blocked (blocking solution: Tris-buffered saline, 1% bovine serum albumin) for 1 h at 25 °C, washed again (5 $\times$), and then incubated with a 1:500 dilution of anti-FLAG M2 antibody in blocking solution for 1.5 h at 25 °C. After another wash (5 $\times$), plates were incubated with horseradish peroxidase-conjugated anti-mouse antibodies diluted 1:5000 in blocking solution. Finally, wells were washed 5 $\times$, and the presence of horseradish peroxidase was assayed by the addition of the chromogenic substrate 3,3',5,5'-tetramethylbenzidine for 5 min. The reaction was stopped by adding an equal volume of 0.5 M H₂SO₄, and color was measured in a plate reader at 450 nm. The absolute concentration of GDNF in purified and conditioned medium samples was determined for the G-hf-GDNF construct using the GDNF E-MAX enzyme-linked immunosorbent assay kit following the manufacturer's instructions (Promega).

Receptor Activation, Complex Formation, Cell Proliferation, and Differentiation Assays—Receptor activation assays were performed as described previously (4). RET-3T3 cells were plated at 85,000 cells/well in 12-well plates and transfected using Superfect (Qiagen) with the reporter plasmids (250 ng/well Gal4-Luc, 50 ng/well Gal4-Elk), CMV-lacZ (50 ng/well) for transfection normalization, a CMV-GFR α (250 ng/well) expression plasmid, a mutant GFL construct (250 ng/well), and 650 ng/well pBluescript as a carrier for a total of 1.5 μ g of DNA/well. Cells were switched to 0.5% serum-containing medium the morning after transfection and harvested 36 h later. The average luciferase activity of duplicate or triplicate samples was normalized to β -galactosidase ac-

tivity of the cotransfected lacZ reporter. For the receptor complex formation assay, Neuro2a cells were transiently transfected using Superfect with an expression plasmid for rat GFR α 1 with a FLAG tag inserted by polymerase chain reaction mutagenesis after the signal sequence. Cells were treated with 25 ng/mL of the indicated factor for 6 h; the cells were washed once with cold phosphate-buffered saline, lysed, and immunoprecipitated using an anti-FLAG M2 monoclonal antibody conjugated to agarose (Sigma). After washing, the immunoprecipitated samples were visualized by immunoblotting with an anti-RET antibody (C-9, Santa Cruz Biotechnology). The NBL-S proliferation assay and the SH-SY5Y differentiation assay were performed as described (4).

Primary Cultured Neuron Survival Assays—Rat cerebellar granule cell dissection and culture was performed as described previously (24). Timed pregnant Harlan Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). At postnatal day 7, cerebella were dissected, cut into 1-mm pieces, and incubated for 15 min in 0.3 g/ml trypsin (Worthington) at 37 °C. The pieces were triturated with a fire-polished Pasteur pipette in the presence of trypsin, and the resulting cell suspension was passed through a Nitex filter (size 3–20/14; Tetko, Elmsford, NY). Cells were plated at a density of 2.3×10^5 cells/cm² in 4-well dishes (Nunc) coated with 0.1 mg/ml poly-L-lysine. Plating medium (K25 + S) consisted of basal medium Eagle (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum, 20 mM KCl, 100 unit/ml penicillin and 100 μ g/ml streptomycin. To reduce the number of non-neuronal cells, 3.3 μ g/ml aphidocholine was added to the medium 24 h after plating.

Granule cell transfections were performed using a modified calcium phosphate protocol as described previously (25). Expression plasmids for GFR α 1, GFR α 2, and RET are described elsewhere (11). At 5 days *in vitro*, medium was replaced with Dulbecco's modified Eagle's medium (Life Technologies, Inc.) for 1 h. During this time, an equal volume of solution containing 0.25 M CaCl₂ and 67 μ g/ml DNA was added to a 2 $\times$ HEPES-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄·7H₂O (Fisher), 15 mM dextrose, and 42 mM HEPES (free acid), pH 7.07) and incubated in the dark at room temperature for 25 min. 30 μ L of the precipitate (1 μ g of DNA) were added to each 4-well dish and incubated at 37 °C for 1 h. Cells were washed twice with Dulbecco's modified Eagle's medium and then returned to plating medium. Transfection efficiency was approximately 0.2–0.5%.

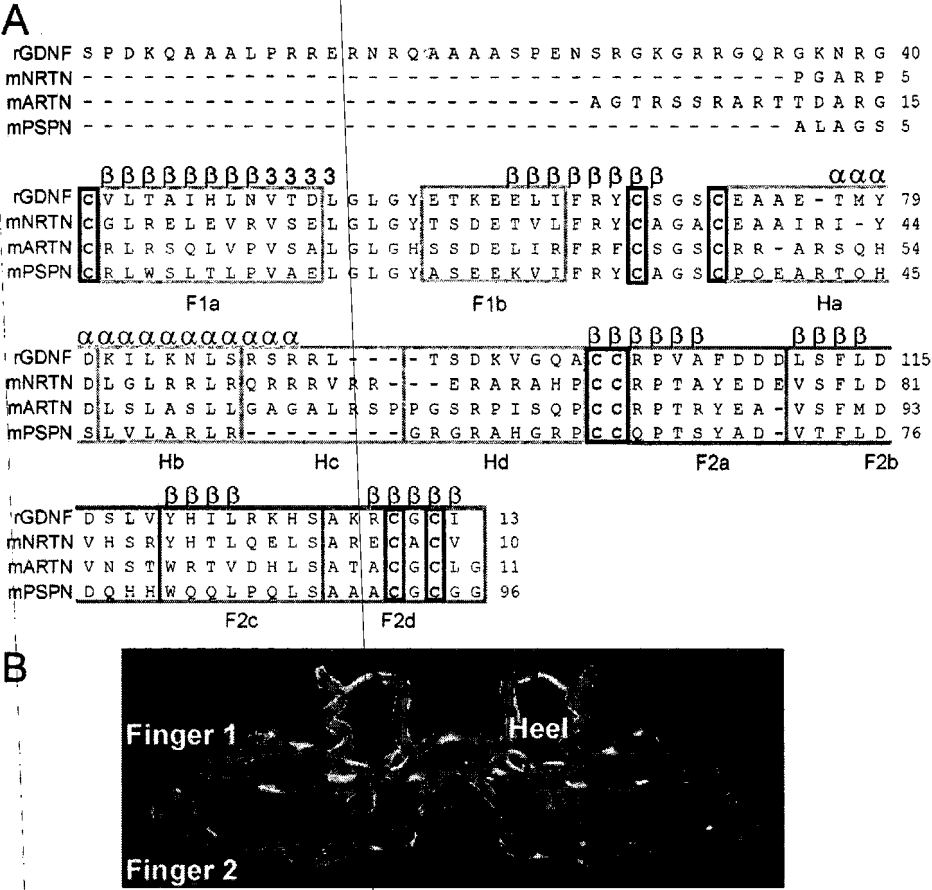
To quantify transfection results, the number of initial EGFP-positive cells in a defined area (minimum of 150 cells) of two to four wells (of a 4-well dish) per condition were counted 24 h after transfection, then rinsed the cultures twice in Dulbecco's modified Eagle's medium and switched them to high potassium/serum (K25 + S), low potassium/no serum (K5-S) medium alone, or K5-S supplemented with the indicated factors for 48 h. After this period, the number of enhanced green fluorescent protein-positive cells remaining in the originally defined area of the well was counted again to obtain the percentage neuronal survival.

Sequence Analysis and Molecular Visualization—Amino acid sequence comparison and alignment was performed using the MegAlign program of the DNASTAR software package. Molecular modeling and analysis was done using the Swiss PDB viewer (26), and models were rendered using Mole software (Applied Thermodynamics). The GDNF structure was modeled after entry 1AGQ (21) of the Protein Data Bank. The "C/D" dimer from 1AGQ is shown, with the missing segment of monomer "C" built as determined for monomer "D."

RESULTS

The N-terminal Extension of GDNF Is Not Required for Activity—An alignment of the mature rat GDNF with mouse NRTN, ARTN, and PSPN is shown in Fig. 1. Rat GDNF was the basis for all mutagenesis in this study because its structure has been determined (21). The largest notable difference between the GFLs is the N-terminal extension before the first structural cysteine, which varies from 40 amino acids in GDNF to only 5 amino acids in NRTN and PSPN, and shows little similarity between the different family members. Furthermore GDNF, NRTN, and ARTN have multiple RXXR consensus subtilisin-like proprotein convertase cleavage sites (27), and therefore multiple isoforms of these extensions are possible. The ones shown are consistent with N-terminal sequencing of GDNF and NRTN (2, 3), the single cleavage site in PSPN (5),

FIG. 1. Primary sequence alignment of the mature GFLs. A, rat GDNF is shown with mouse NRTN, ARTN, and PSPN. Secondary structure elements ("α" for α-helix, "β" for β-strand, and 3 for 3₁₀-helix) as determined for GDNF (21) are indicated above the alignment. The regions delineated for homologue-scanning mutagenesis are in colored blocks, with the name of the region ("F1a-b" for finger 1, "Ha-d" for heel, and "F2a-d" for finger 2) below the given block. The color of each block corresponds to the color scheme shown in B. B, representation of backbone of the GDNF dimer showing the first (blue) and second (red) fingers and the heel region (green) of the molecule.



and a cleavage site conserved between mouse and human ARTN (4).

To determine if the large N-terminal extension of GDNF is required for its activity, we took advantage of the differential processing of GDNF and NRTN by mammalian cells. Chimeric constructs with the prepro-region from NRTN attached to GDNF were generated, and we assessed both their processing and their ability to activate the GFRα1-RET receptor complex (Fig. 2). Tandem 6X histidine and FLAG tags were inserted after the RXXR cleavage site of GDNF or NRTN to allow purification and tracking of the proteins. When expressed in COS cells, similar quantities of tagged GDNF with its own pro-region (G-hf-GDNF) or the NRTN pro-region (N-hf-GDNF) could be purified from conditioned medium using nickel chromatography (Fig. 2B). Only species corresponding to the expected processing events were observed in the medium, indicating that the pro-domains of NRTN and GDNF are sufficient to direct proper processing of their different N-terminal extensions.

To assess the ability of these proteins to activate the high affinity GDNF receptor, GFRα1-RET, we utilized the Gal4-Elk1/Gal4-luciferase reporter system as described previously (4). This system monitors the level of RET activation of the mitogen-activated protein kinase pathway, which induces the Gal4-Elk1 fusion protein to activate the Gal4-luciferase reporter, giving a facile measure of receptor activation (28, 29). The reporter plasmids, together with expression plasmids for GFRα1 and the indicated GDNF construct, were cotransfected into fibroblasts that stably express RET (Fig. 2C). Subsequently, the steady-state level of GFRα1-RET activation was measured after two days of autocrine ligand stimulation by the cells. Wild-type GDNF, G-hf-GDNF, and N-hf-GDNF all showed comparable activation of GFRα1-RET in this assay, whereas tagged PSPN constructs with either the PSPN or

NRTN pro-region did not activate the receptor. These results are consistent with previous receptor activation and binding experiments indicating that GDNF, but not PSPN, can bind and activate RET through GFRα1 (4, 5). Furthermore, these data indicate that the N-terminal extension of GDNF is not required for its ability to activate the GFRα1-RET receptor complex, and the location of the epitope tag does not interfere with the function of GDNF.

Homologue-scanning Mutagenesis Identifies Regions of GDNF Critical for Interacting with GFRα1 and GFRα2—Although the crystal structure of GDNF is known, the molecular determinants for GDNF-GFRα receptor binding and specificity are currently undefined. To identify regions of GDNF that are required for its ability to activate the GFRα1-RET receptor complex, we utilized homologue-scanning mutagenesis together with the receptor activation assay described above. Ten GDNF mutants (termed "GPG" mutants) were generated by replacing blocks of nonconserved sequence from PSPN into GDNF (Fig. 3, see Fig. 1 for sequence changes). This method allows comprehensive mutagenesis of sites responsible for the difference in the ability of GDNF and PSPN to activate GFRα1-RET and is highly likely to maintain structural integrity of the mutants because the replacements are from a homologous (and likely structurally similar) protein. All mutants were produced at similar quantities when transfected into COS cells, and the processing of each appeared normal (Fig. 3B).

Comparison of the GPG mutants with GDNF revealed that although many mutants showed slightly decreased activity, only one (GPG-F2a) completely lost the ability to activate the GFRα1-RET receptor (Fig. 4A). This region maps to the bottom of the GDNF molecule along the second finger (Fig. 4B) (21). The only other notable mutant that consistently showed activity 60% or lower than full-length GDNF was GPG-F2c, which

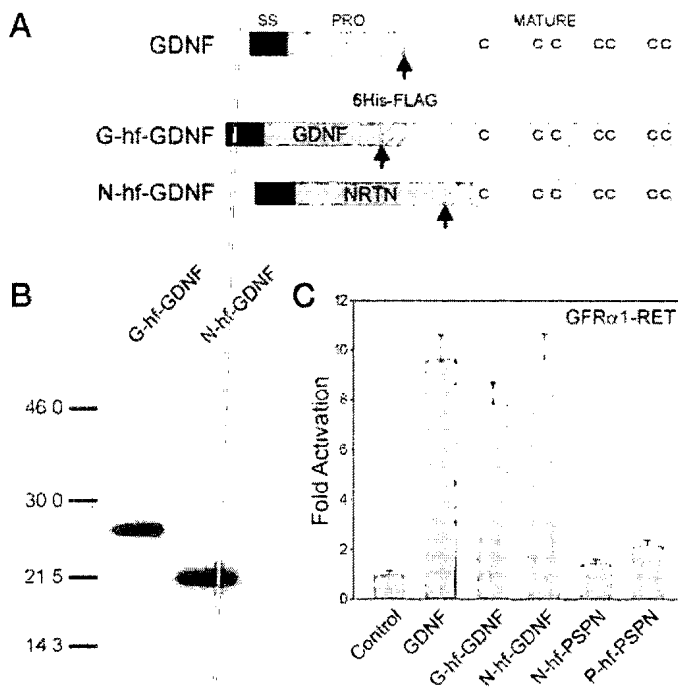


FIG. 2. The N-terminal extension of GDNF is not required for bioactivity. A, schematic diagram of wild-type and tagged GDNF constructs. The site of pro-protein convertase cleavage of GDNF is indicated by the arrow (SS, signal sequence; PRO, pro-domain; MATURE, mature processed monomer). GDNF with a 6-histidine and FLAG tag inserted two residues after the RXXR cleavage site is designated "G-hf-GDNF." To generate GDNF lacking the N-terminal extension, a chimera was produced with the NRTN prepro-region attached to GDNF truncated two residues upstream of the first structural cysteine. B, production of tagged GDNF proteins in COS cells. The indicated construct was transiently transfected into COS cells; recombinant protein was purified from conditioned medium using nickel chromatography and visualized on an anti-FLAG immunoblot. Monomeric G-hf-GDNF ran at ~25 kDa, and N-hf-GDNF at ~21.5 kDa, consistent with processing at the predicted RXXR cleavage sites from GDNF and NRTN. Therefore N-hf-GDNF produces GDNF with the 40-amino acid N-terminal extension replaced by two amino acids and the tandem His-FLAG tags. C, comparison of GFRα1-RET receptor activation by wild-type and tagged GDNF constructs. RET-expressing fibroblasts were cotransfected with GFRα1, the reporter plasmids (Gal4-Elk1 and Gal4-luciferase), and the indicated GDNF expression construct. Reporter activation was measured after 36 h of autocrine ligand stimulation by the cells and reported as fold activation over the control condition. Mean and S.D. are shown for duplicates from a representative experiment. Tagged full-length and truncated GDNF showed similar receptor activation to wild-type GDNF, whereas tagged PSPN with its own (P-hf-PSPN) or NRTN's pro-region (N-hf-PSPN) does not activate GFRα1-RET.

maps to the strand adjacent to the region F2a along the bottom of the molecule.

It is well established that GDNF is also capable of binding and activating the GFRα2-RET receptor, albeit with a lower affinity than NRTN (11, 15, 16), whereas PSPN cannot (5, 13). Therefore, to compare the molecular determinants of GDNF required for GFRα1 versus GFRα2 interaction, we tested the ability of this same panel of mutants to activate the GFRα2-RET receptor complex. Several of the GPG mutants were significantly more attenuated in their ability to activate RET through GFRα2 than they were through GFRα1 (Fig. 4C). As before, mutant GPG-F2a was unable to activate the receptor. Interestingly, mutant GPG-F2c, which was the second most affected mutant in activating GFRα1-RET, showed essentially no activation of GFRα2-RET. Finally, the mutant that showed the most contrast in its ability to activate GFRα1-RET versus GFRα2-RET was mutant GPG-Ha, which was severely affected only in its ability to activate GFRα2-RET. Interestingly, mapping these three regions to the GDNF crystal struc-

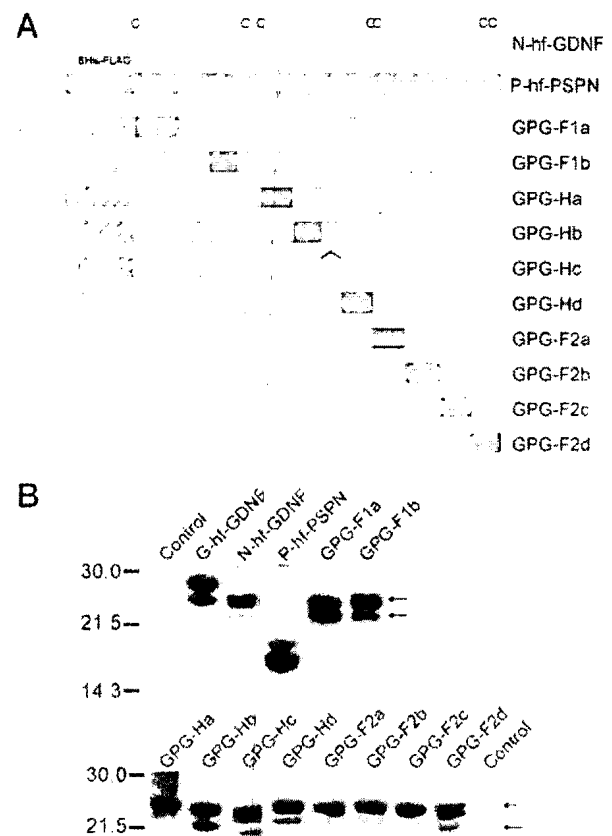


FIG. 3. Homologue-scanning mutagenesis of GDNF. A, schematic representation of mature region from GDNF, PSPN, and the homologue-scanning mutants. Mutant GPG-Hc is a deletion of amino acids 88–92 of mature GDNF to correspond with the lack of these residues in PSPN. The sequence replacements in these mutants are shown in Fig. 1. B, anti-FLAG immunoblot of total cell lysates from COS cells transfected with the different homologue scanning mutant constructs. Mutants were produced in similar quantities. The two major bands indicated by arrows represent the unprocessed and mature forms of the mutants. The lower band corresponds in size to the secreted product (see Fig. 2B). The smaller size of PSPN than N-hf-GDNF reflects the fact that GDNF is glycosylated (2), whereas PSPN is not.

ture reveals that they form a continuous surface, with residues from F2a and F2c from one monomer directly adjacent to region Ha from the heel of the other monomer, suggesting a possible receptor-ligand interaction surface (Fig. 4D). In summary, the above mutagenesis data suggest that two adjacent regions of GDNF located along the second finger are necessary for interaction with both GFRα1 and GFRα2 (regions F2a and F2c) and that additional regions appear to be required for GDNF interaction with GFRα2.

Identification of Regions F2a and F2c from GDNF, NRTN, or ARTN as Sufficient for Activating the GFRα1-RET Receptor Complex—The homologue-scanning screen above suggests several regions that are necessary for the full ability of GDNF to activate the GFRα1-RET receptor. However, using only loss of function analysis it is difficult to assess whether critical receptor-contact residues are being altered directly or if the mutations induce structural changes that alter receptor contact surfaces elsewhere on the molecule. To identify if any of the critical regions identified above (either alone or in combination) are sufficient to allow binding and activation of the GFRα1-RET receptor complex, we generated PSPN mutants with the putative critical regions from GDNF replacing the corresponding regions of PSPN. We then determined if these "PGP" mutants had gained the ability to activate the GFRα1-RET receptor complex (Fig. 5).

We focused initially on two candidate regions, F2a and F2c

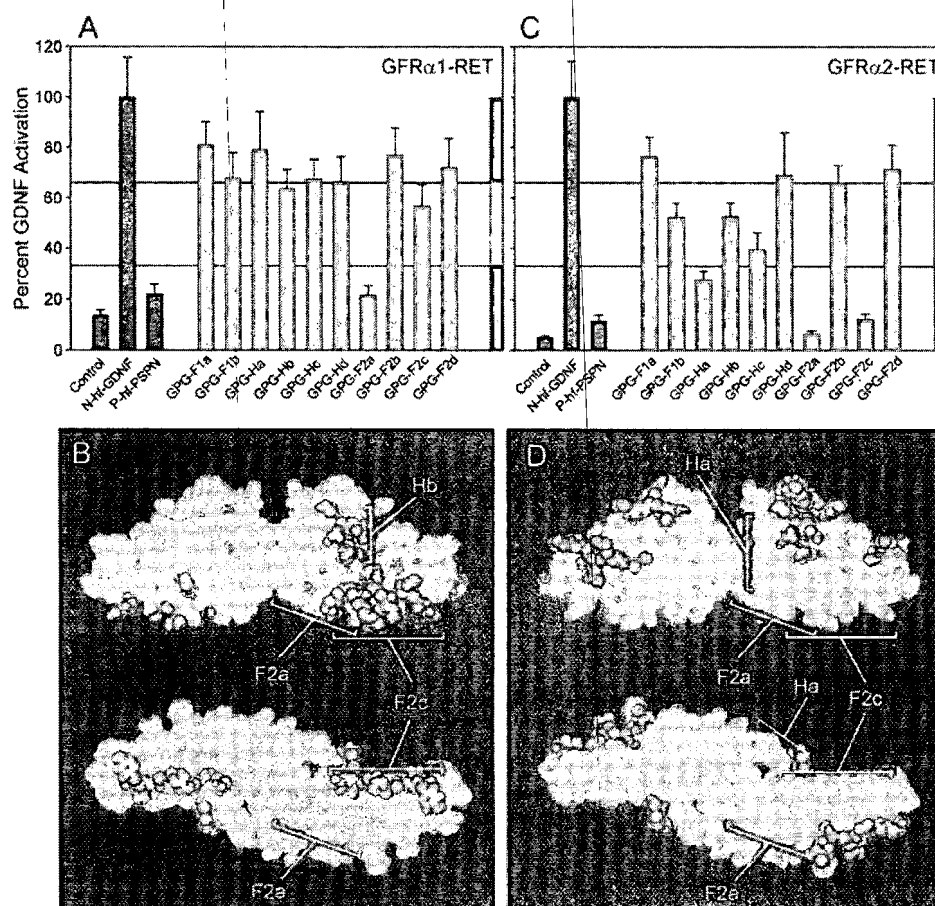


FIG. 4. Receptor activation analysis of homologue-scanning mutants. A, GFRα1-RET receptor activation by homologue-scanning GDNF mutants. Values are represented as a percentage of reporter activation by GDNF. The mean and standard deviation of triplicate measurements from a representative experiment are shown. Similar results were obtained from three other experiments. Mutant GPG-F2a was severely attenuated in its ability to activate GFRα1-RET. All other mutants showed slightly decreased activity at 70–80% of GDNF. The only other mutant that consistently showed receptor activation below 60% of GDNF was GPG-F2c. Arbitrary boundary lines at 33 and 66% of wild-type GDNF activation are designated in color to simplify visualization in B. All mutants in the yellow and red regions were significantly less active than full-length GDNF ($p < 0.05$, Student's paired t test). B, location of putative critical GFRα1-RET interaction domains identified in A on a space-filling model of the GDNF crystal structure (21). Regions are colored green above 66%, yellow below 66%, and red below 33% of GDNF activation. The upper representation is a side view of the GDNF dimer, and the lower representation is from a viewpoint below the dimer. C, GFRα2-RET receptor activation by homologue-scanning mutants. The mean and standard deviation of duplicate measurements from a representative experiment are shown. Similar results were obtained from three other experiments. As in A, all mutants in the yellow and red regions were significantly less active than full-length GDNF ($p < 0.05$, Student's paired t test). Mutants GPG-F2a and GPG-F2c were both severely attenuated in GFRα2-RET activation, similar to control and PSPN. Three additional mutants were more attenuated in their ability to activate GFRα2-RET than GFRα1-RET (F1b, Hb, Hc). Finally, mutant GPG-Ha was significantly less effective at activating GFRα2-RET (~27%) than GFRα1-RET (~80%). D, location of putative critical GFRα2-RET interaction domains identified in C. Color scheme and viewpoints of structure are same as for B.

along the second finger of GDNF, because mutant GPG-F2a was entirely inactive, and mutant GPG-F2c was the only other mutant that consistently showed decreased activation of GFRα1-RET. Replacement of either region F2a or F2c alone from GDNF into PSPN was not sufficient to allow the resulting mutants (PGP-F2a or PGP-F2c) to activate GFRα1-RET. However, when both regions were placed into PSPN the resulting mutant (PGP-F2ac) gained the ability to activate the GFRα1-RET receptor at a level comparable to full-length GDNF (Fig. 5B). Furthermore, treatment of GFRα1-transfected Neuro2a cells (which endogenously express Ret) with either GDNF or mutant PGP-F2ac induced comparable GFRα1-RET complex formation, demonstrating that PGP-F2ac had gained the ability to bind and induce receptor complex formation (Fig. 5C). To determine if mutant PGP-F2ac was also able to elicit biological responses comparable to GDNF, we tested its ability to induce proliferation in the NBL-S neuroblastoma cell line, which expresses GFRα1 and RET and responds to GFRα1-RET agonists (GDNF, NRTN, ARTN) by proliferation (Fig. 5D) (4). Mutant PGP-F2ac purified from transiently transfected COS cell-conditioned medium induced robust

proliferation of NBL-S cells, similar to GDNF at all doses, whereas PSPN did not. Furthermore, PGP-F2ac was also able to induce neurite outgrowth in the GFRα1-RET-expressing neuroblastoma line SH-SY5Y similar to GDNF (Fig. 5E) (4).

NRTN and ARTN are also able to activate GFRα1-RET (4, 30), and therefore we examined if the same regions (F2a and F2c) from these two additional GFRα1 agonists are also sufficient to activate the GFRα1-RET receptor (Fig. 6). Interestingly, the corresponding PSPN mutants with regions F2a and F2c from NRTN and ARTN were also capable of activating the GFRα1-RET receptor complex, at levels comparable to GDNF and PGP-F2ac (Fig. 6B). This indicates that elements of regions F2a and F2c from all the known GFRα1 agonists are sufficient to activate GFRα1-RET when placed in the context of PSPN. Furthermore, these regions appear to be sufficient only for GFRα1-RET activation, as the same mutants did not activate GFRα2-RET comparably to GDNF (Fig. 6C). Mutants PNP-F2ac and PGP-F2ac did elicit low level activation of GFRα2-RET, whereas PAP-F2ac did not. This is consistent with the ability of GDNF and NRTN, but not ARTN, to activate GFRα2-RET (4) and indicates that regions F2a and F2c are also

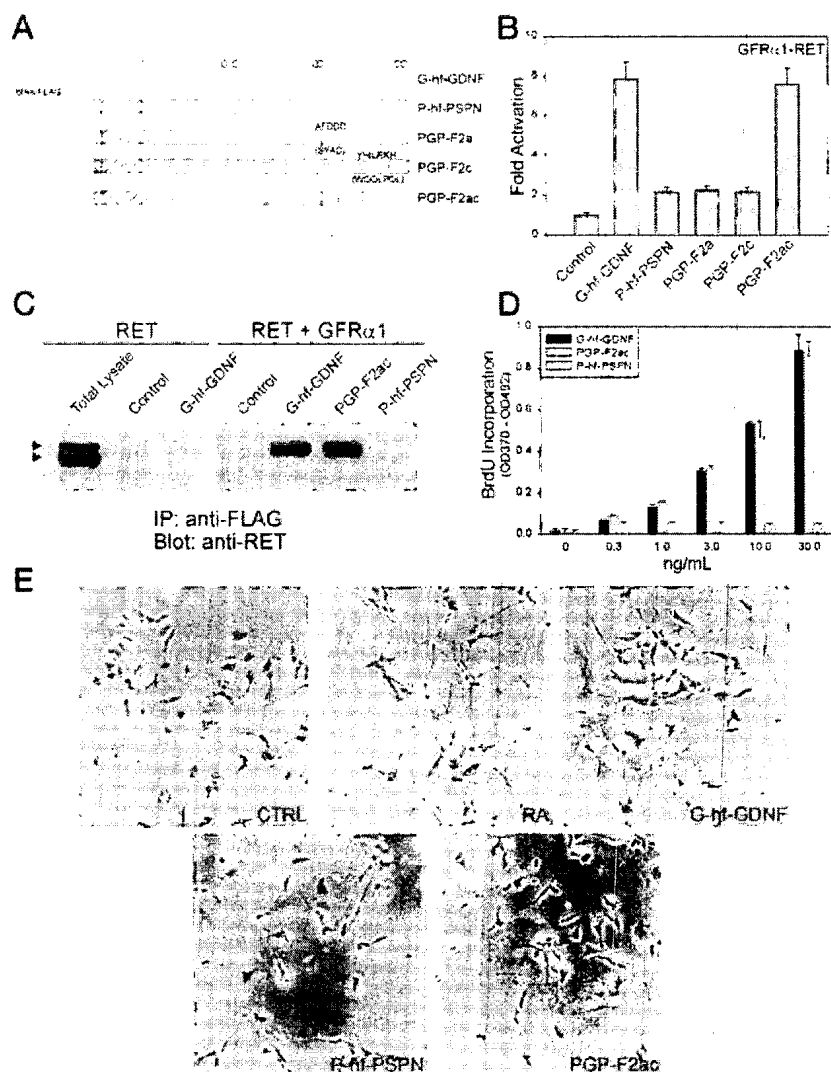


FIG. 5. GDNF/PSPN chimeras identify regions F2a and F2c from GDNF as sufficient for GFR α 1-RET activation and bioactivity on GFR α 1-RET-expressing cells. *A*, schematic diagram of GDNF/PSPN replacement chimeras (PGP) for gain-of-function analysis. The PSPN amino acid sequence in parentheses below the schematics was replaced by a GDNF sequence, shown above. *B*, both regions F2a and F2c from GDNF are required for activating GFR α 1-RET in the context of PSPN, as measured by the luciferase-based reporter assay. *C*, mutant PGP-F2ac induces GFR α 1-RET receptor complex formation. Neuro2a cells either untransfected (RET) or transfected with FLAG-tagged GFR α 1 (RET-GFR α 1) were treated with the indicated factor at 25 ng/mL. Cells were lysed, immunoprecipitated with an anti-FLAG antibody, and immunoblotted using an anti-RET antibody. A fraction of the total lysate shows the RET doublet, with the upper band corresponding to the mature cell-surface form. As expected, treatment of nontransfected Neuro2a cells (that express only RET) with GDNF does not lead to complex formation. However, GFR α 1-transfected Neuro2a cells treated with GDNF or PGP-F2ac, but not PSPN, results in RET coimmunoprecipitating with GFR α 1. *IP*, immunoprecipitate. *D*, histogram of bromodeoxyuridine (BrdU) incorporation by NBL-S neuroblastoma cells in the presence of the indicated factors purified from conditioned medium of transiently transfected COS cells. Mutant PGP-F2ac stimulates proliferation of NBL-S cells at a similar level to GDNF at all doses tested. *E*, SH-SY5Y neuroblastoma cells were cultured in the presence of no factor (CTRL), retinoic acid (RA, 10 μ M), or conditioned medium from COS cells expressing the indicated construct. GDNF and PGP-F2ac stimulated neurite outgrowth in these cells, whereas PSPN did not.

involved in binding and activating GFR α 2-RET but that additional regions are required for full activation.

Additional Determinants Are Critical for NRTN to Activate GFR α 2-RET and for ARTN to Activate GFR α 3-RET—The observation that mutants PGP-F2ac and PNP-F2ac cannot fully activate GFR α 2-RET is consistent with the homologue-scanning mutagenesis above (Fig. 4), which suggested that regions in addition to F2a and F2c are required for GDNF to activate GFR α 2-RET. In particular, mutant GPG-Ha was significantly attenuated in its ability to activate GFR α 2-RET (~27%), whereas it showed a minor loss in the ability to activate GFR α 1-RET (~80%, Fig. 4). To address whether region Ha contains additional molecular determinants required for activating GFR α 2-RET, we generated a PSPN mutant with regions F2a, F2c, and Ha replaced by the corresponding regions from NRTN, the highest affinity GFR α 2 agonist (PNP-Ha/F2ac, Fig. 7A). Whereas mutant PNP-F2ac elicited only a minor increase in GFR α 2-RET activation as before, mutant PNP-Ha/F2ac was

significantly better at activating GFR α 2-RET; however, it did not restore full activity (usually 70–80% of GDNF, Fig. 7B). Therefore, consistent with the homologue-scanning mutagenesis above, these data indicate that regions F2a and F2c of GDNF and NRTN are only sufficient to activate GFR α 1-RET, and additional regions are required for full activation of GFR α 2-RET (region Ha and perhaps others).

ARTN is a recently identified member of the GFLs that can activate GFR α 1-RET, and the only member of the family that can activate GFR α 3-RET (4). As shown above, regions F2a and F2c from ARTN are sufficient to activate GFR α 1-RET. In an initial attempt to characterize the molecular determinants of the ARTN-GFR α 3 interaction, we examined the ability of PSPN-ARTN chimeras to activate the GFR α 3-RET receptor complex (Fig. 7, C and D). As expected, N-hf-ARTN gave robust activation of GFR α 3-RET, whereas N-hf-GDNF showed no activity. Mutant PAP-F2ac, which is capable of activating GFR α 1-RET (see Fig. 6), was also unable to activate

GFR α 3-RET, indicating that like the NRTN-GFR α 2 interaction, the ARTN-GFR α 3 interaction requires regions in addition to F2a and F2c. To determine if region Ha was also involved in the ARTN-GFR α 3 interaction, we generated a PSPN mutant with regions Ha, F2a, and F2c from ARTN (Fig. 7C). Interestingly, whereas mutant PAP-F2ac was entirely inactive, mutant

PAP-Ha/F2ac regained significant activity (usually 70–80% of full length ARTN, Fig. 7D). Therefore, similar to the NRTN-GFR α 2 interaction, the ARTN-GFR α 3 interaction requires molecular determinants from both the heel and finger 2 regions of the molecule, whereas interaction of GDNF, NRTN, or ARTN with GFR α 1 only requires regions F2a and F2c from finger 2.

PSPN/GDNF Chimeras Function as GFR α 1-specific Agonists in Vitro—The homologue-scanning mutagenesis and gain of function experiments above demonstrate that for the GFR α 1-RET agonists (GDNF, NRTN, and ARTN), regions F2a and F2c are sufficient for interaction with GFR α 1 but that additional regions are required for interaction with GFR α 2 and GFR α 3 (Figs. 6 and 7). This suggests that the PSPN mutants containing only regions F2a and F2c from GDNF, NRTN, or ARTN may function as GFR α 1-RET-specific agonists. To test this idea, we developed a cell survival assay using rat cerebellar granule cells in culture. These cells do not express endogenous RET or GFR α coreceptors and can be transfected efficiently, therefore providing a model system of cell survival with defined receptor components. Cerebellar granule cells transfected with RET or GFR α 1 alone do not survive in the absence of high potassium or in the presence of GDNF; however, cells cotransfected with GFR α 1 and RET survive in the presence of GDNF at levels comparable to the control condition of high potassium plus serum (Fig. 8A) (24). Consistent with other assays, cerebellar granule cells transfected with GFR α 1-RET or GFR α 2-RET survive in the presence of either GDNF or NRTN, but not PSPN (Fig. 8B). However, mutant PGP-F2ac was only capable of supporting the survival of GFR α 1-RET-transfected, but not GFR α 2-RET-transfected cells. This is consistent with its minimal ability to activate the GFR α 2-RET receptor in fibroblasts (Fig. 6C) and indicates that unlike GDNF and NRTN, mutant PGP-F2ac functions as a GFR α 1-RET-specific agonist in this *in vitro* cell survival paradigm.

DISCUSSION

We report a comprehensive mutagenesis analysis of GDNF, the prototypical member of the GDNF family of neurotrophic

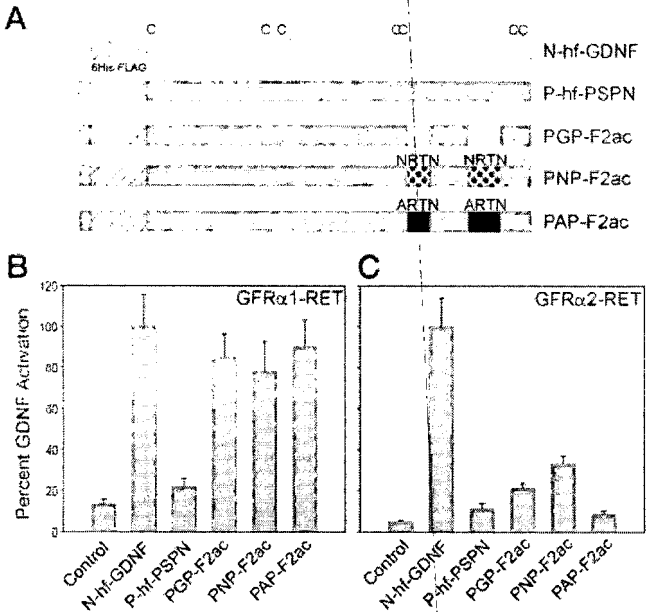


FIG. 6. Regions F2a and F2c from NRTN and ARTN are also sufficient to activate GFR α 1 but not GFR α 2. A, schematic diagram showing NRTN/PSPN and ARTN/PSPN replacement chimeras. B, mutants PNP-F2ac and PAP-F2ac can also activate GFR α 1-RET at similar levels to GDNF or PGP-F2ac. In contrast, none of these mutants (PGP-F2ac, PNP-F2ac, PAP-F2ac) were capable of activating GFR α 2-RET at levels similar to GDNF. Mutants PGP-F2ac and PNP-F2ac showed low level activation of GFR α 2-RET, whereas PAP-F2ac did not, consistent with the ability of GDNF and NRTN, but not ARTN, to activate GFR α 2-RET.

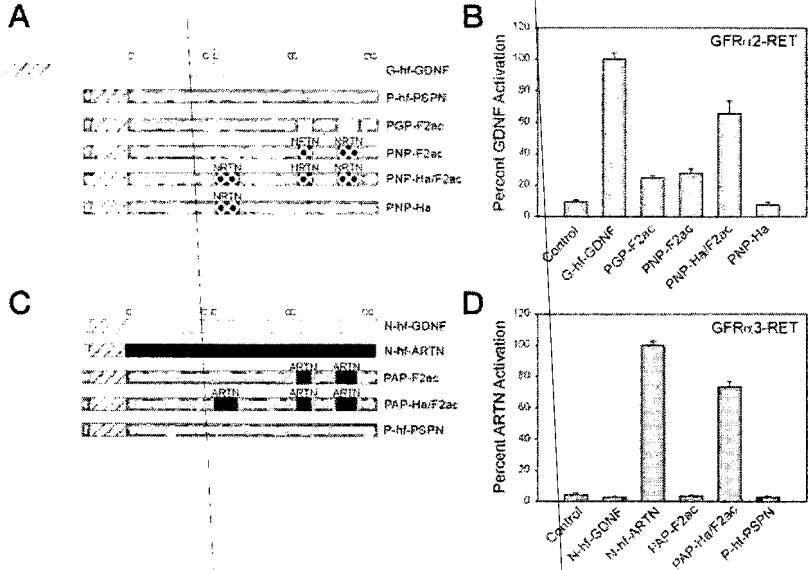


FIG. 7. Region Ha is also involved in GDNF or NRTN interaction with GFR α 2 and ARTN with GFR α 3. A, schematic of NRTN/PSPN replacement mutants. B, replacement of regions F2a, F2c, and Ha together from NRTN into PSPN restores activity to ~70% (PNP-Ha/F2ac), significantly greater than regions F2a and F2c alone. This is consistent with the homologue-scanning mutagenesis above (Fig. 4), which suggested that region Ha is also required for GDNF interaction with GFR α 2. As expected, region Ha alone from NRTN was not sufficient to activate GFR α 2-RET (PNP-Ha). C, schematic of ARTN/PSPN chimeras used to delineate requirements for GFR α 3 activation. D, as reported previously, ARTN activates the GFR α 3-RET receptor, whereas GDNF cannot. Mutant PAP-F2ac was also not capable of activating GFR α 3-RET, indicating that like the NRTN-GFR α 2 interaction, the ARTN-GFR α 3 interaction requires regions in addition to those sufficient to activate GFR α 1-RET (F2a and F2c). However, further addition of region Ha (mutant PAP-Ha/F2ac) from ARTN restored significant activity (~70%), indicating region Ha is also critical for the ARTN-GFR α 3 interaction.

factors, in an attempt to characterize at the molecular level the interaction between GDNF and its receptors GFR α 1 and GFR α 2. This analysis revealed similar regions are sufficient for GFR α 1 interaction by all known GFR α 1 ligands; GDNF, NRTN, and ARTN. Furthermore we found that additional regions are required for activating GFR α 2-RET and GFR α 3-RET, which allowed the production of GFR α 1-RET-specific agonists as tested in receptor activation and cell survival assays.

The N-terminal Extension of GDNF Is Not Required for Activity—The N-terminal extension before the first structural cystine-knot cysteine is highly variable among the TGF- β superfamily members. In the case of TGF- β 2, it exists as a short α -helix and is stabilized by an additional disulfide bond relative to other members of the superfamily (19, 20). However, in GDNF and OP-1, the only other members of the TGF- β superfamily that have been crystallized, the 35–37 residue N-terminal extension was unresolved and therefore does not adopt a consistent conformation in solution (21, 31). We found that truncating the N-terminal extension did not affect the ability of GDNF to activate its receptors. This is consistent with experiments characterizing monoclonal antibodies against GDNF that also suggested the N-terminal extension is not required for receptor binding or bioactivity (32). The role of the N-terminal extension of GDNF remains unclear; however, because it does not appear to be involved in receptor interaction, possibilities such as its importance for stability and/or binding additional proteins or to extracellular matrix elements warrant consideration.

Characteristics of the Putative GFR α Interaction Site of GDNF—The mutagenesis reported here indicates that critical residues for GFR α receptor interaction and specificity are located in the second finger region of the GDNF molecule. However, whereas the use of homologue-scanning mutagenesis is ideally suited for identifying sites involved in differential receptor specificity, it cannot delineate all residues involved in receptor binding, as some of these may be identical in GDNF and PSPN. The fact that residues from regions F2a and F2c are sufficient when placed in the context of PSPN to activate GFR α 1-RET indicates that residues in these regions are likely to be directly involved in binding to GFR α s. Although it is possible that the GFLs also contact RET in the active receptor complex, the fact that all members of the GFLs, including PSPN, appear to signal through RET makes it doubtful that the regions identified by our mutagenesis scheme are involved in RET interaction directly (4, 14). Because we focused on functional activation of the GFR α -RET receptor complex rather than receptor binding assays, we cannot exclude the possibility that some of the nonfunctional mutants produced here may still be capable of binding to the GFR α coreceptors. Such mutants could potentially function as receptor antagonists, and we are currently investigating this possibility.

Current evidence suggests that the stoichiometry of the active receptor complex for GDNF is (GDNF) $_1$ (GFR α) $_2$ (RET) $_2$ (33). Residues from regions F2a and F2c project essentially from the bottom the GDNF structure and form symmetric sites on both monomers of the molecule (see Fig. 4B). Interestingly, residues from region Ha (which is critical for GDNF-GFR α 2 and ARTN-GFR α 3 interaction) lie directly adjacent to regions F2a and F2c from the finger 2 region of the opposing monomer and form continuous surfaces along the side and bottom of the molecule (see Fig. 4C). Because these regions are all critical for GFR α specificity, we suggest that the molecular surface formed by the heel of one monomer and the second finger of the adjacent monomer form GFR α binding surfaces on GDNF.

Comparison of structure-function studies from other TGF- β and cystine-knot superfamily members reveals common themes in the location of receptor binding surfaces. The best

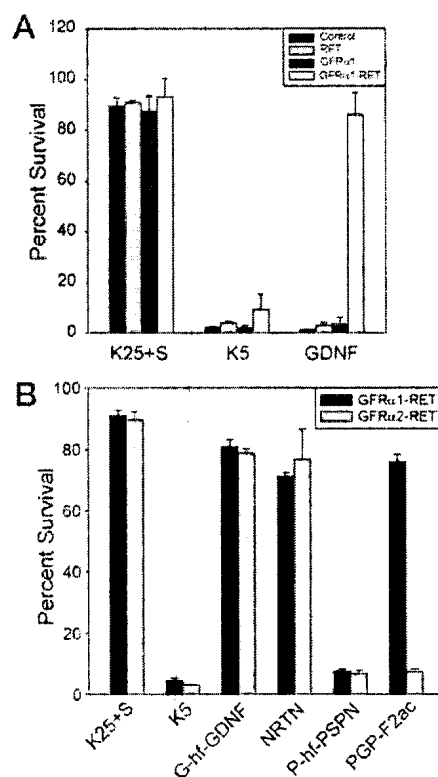


FIG. 8. Mutant PGP-F2ac functions as a GFR α 1-RET-specific agonist in a neuronal survival assay. A, cerebellar granule cells dissected from 5-day-old rats were transfected with the indicated receptor components and cultured for 3 days in the presence of high potassium plus serum (K25+S), low potassium (K5), or low potassium plus GDNF (GDNF). Only neurons transfected with GFR α 1 and RET together survived in the presence of GDNF. B, cerebellar granule cells transfected with GFR α 1-RET or GFR α 2-RET were cultured in the presence of the indicated factors. NRTN was bacterially produced and added at 50 ng/mL. All others were produced in COS cells and added as concentrated conditioned medium. Whereas GDNF and NRTN support the survival of neurons expressing GFR α 1-RET or GFR α 2-RET similarly, mutant PGP-F2ac only supports the survival of GFR α 1-RET expressing neurons.

characterized of these is the binding of vascular endothelial growth factor with its receptors KDR and Flt-1, where extensive mutagenesis and co-crystallization have been performed (34, 35). Mutagenesis of vascular endothelial growth factor identified a cluster of residues critical for KDR binding, several of which were along the adjacent β -strands of the second finger, similar to regions F2a and F2c identified here for GDNF. Furthermore, even though the orientation of the monomers in the vascular endothelial growth factor dimer is different from GDNF, the receptor binding site on vascular endothelial growth factor involves residues from finger 2 and the analogous heel region of the opposing monomer (34). Finally, mutagenesis of TGF- β indicates that the binding affinity of the different TGF- β isoforms for the TGF- β type II receptor is also determined by residues along the second finger of the molecule, analogous in location to region F2c (36, 37). Therefore it is possible that although the receptor systems and dimerization modes are strikingly different for these different cystine-knot superfamily proteins, the location of their receptor interaction surfaces may be quite similar.

Differences in GFR α 1-GFR α 2-GFR α 3 Interaction—In our analysis of the structural determinants of GDNF required for activating its two functional receptors, GFR α 1-RET and GFR α 2-RET, we observed that only regions F2a and F2c were required for activating GFR α 1-RET but additional regions (including region Ha) were required for activating GFR α 2-RET. GFR α 1-RET is the most promiscuous of the GFL receptors and is able to interact with three of the four known ligands. This is

consistent with GFR α 1-RET having the most minimal requirements for being activated (regions F2a and F2c) and additional regions being required for activating GFR α 2-RET and GFR α 3-RET. It is of note that regions F2a and F2c from GDNF, NRTN, or ARTN conferred similar activity to PSPN in the receptor activation assay. It will be interesting to determine if these mutants confer similar GFR α 1-binding characteristics to their parent constructs (PGP-F2ac with higher affinity than PNP-F2ac or PAP-F2ac) or if they all bind GFR α 1 with similar affinity, suggesting that regions of NRTN and ARTN outside F2a and F2c may actually reduce their affinity for GFR α 1.

Although region Ha is critical for the NRTN-GFR α 2 and ARTN-GFR α 3 interactions, PSPN mutants containing regions Ha, F2a, and F2c were not fully active, suggesting that either additional regions are necessary or the binding determinants from NRTN and ARTN are not presented properly in the context of the PSPN molecule. A better understanding of receptor complex formation and stoichiometry and direct co-crystallization will be required to confirm and refine our understanding of the receptor binding surfaces of these molecules.

Utility of GFL Chimeras as GFR α 1-specific Agonists—The GFLs are promising candidates as therapeutic agents for the treatment of neurodegenerative diseases, including Parkinson's disease. GDNF, NRTN, and ARTN all support the survival of cultured dopaminergic neurons of the embryonic ventral midbrain (2, 4, 6), and GDNF and NRTN have both shown to be effective in supporting the survival of midbrain dopaminergic neurons in animal injury models (6, 10). Several lines of evidence argue that the effects of GDNF, NRTN, and ARTN on dopaminergic ventral midbrain neurons are mediated through the GFR α 1-RET receptor system. First, because GFR α 3 is not expressed in the ventral midbrain and ARTN cannot utilize GFR α 2, survival promotion of these neurons by ARTN is likely through its ability to activate GFR α 1-RET (4). Second, GFR α 2 expression is diffuse and weak in the pars compacta region of the substantia nigra and does not colocalize with tyrosine hydroxylase staining neurons, in contrast to the significantly stronger expression of GFR α 1, which does colocalize with tyrosine hydroxylase staining neurons (6). Finally, the ability of both GDNF and NRTN to support the survival of dopaminergic ventral midbrain neurons is lost in GFR α 1 knockout mice, indicating that at least in the embryo the survival promotion of dopaminergic ventral midbrain neurons is only through GFR α 1-RET signaling (38). Because there are several central and peripheral sites of GFR α 2-RET expression that could lead to side effects in the treatment of patients with GDNF or NRTN, the GFL chimeras that function as GFR α 1-RET-specific agonists described here (PGP-F2ac and particularly PAP-F2ac) could be therapeutically useful as they would minimize potential GFR α 2-related side effects, while maintaining the ability to support survival and growth of the desired GFR α 1-RET expressing target neuron populations.

Acknowledgments—We thank Shelly Audrain and Tim Fahrner for their technical support, and Pat Lampe and Krista Molder for their help with primary neuron cultures.

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