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Autocatalytic polysialylation of polysialyltransferase-1

Martina Mühlenhoff, Matthias Eckhardt, Andrea Bethe, Matthias Frosch and Rita Gerardy-Schahn[1]

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 30625 Hannover, Germany

[1]Corresponding author

Polysialic acid (PSA) is a specific and highly regulated post-translational modification of the neural cell adhesion molecule NCAM. Synthesis of PSA depends on the activity of a single enzyme, the polysialyltransferase-1 (PST-1), recently cloned from three mammalian species. The present study was carried out to investigate the catalytic mechanism of PST-1. Using a newly developed *in vitro* assay system, we demonstrate autopolysialylation for PST-1. The synthesis of PSA chains, which involved *N*-glycosylation sites, occurred immediately after contact with the activated sugar donor CMP-Neu5Ac. In contrast to the polysialylation of NCAM, where terminal sialylation in either the α2,3 or α2,6 position is required, the autopolysialylation could be started in the asialo-PST-1 isolated from CHO cells of the Lec2 complementation group. Pre-formed PSA chains were not transferred to NCAM. Nevertheless, the autocatalytic step is likely to be a prerequisite for enzymatic activity, since agalacto-PST-1 isolated from Lec8 cells was functionally inactive. Our data describe a novel route of autocatalytic maturation of a glycosyltransferase and thereby provide a new basis for studies aimed at elucidating and influencing the catalytic functions of PST-1.

Keywords: autocatalysis/NCAM/polysialic acid/polysialyltransferase-1

Introduction

Homopolymers of α2,8-linked *N*-acetylneuraminic acid (polysialic acid; PSA) represent a widely distributed sugar structure occurring from bacteria to man (Troy, 1992). While neural cell adhesion molecule (NCAM) is the most abundant PSA carrier in mammals (Edelman and Crossin, 1991; Rutishauser, 1995), the α-subunit of the sodium channel in rat (Zuber *et al.*, 1992) and a still undefined intracellular protein in breast cancer and leukaemia cell lines (Martersteck *et al.*, 1996) have also been reported to be polysialylated. Numerous reports of the last decade demonstrate PSA to be a major regulator of cell–cell interactions associated with neural development and neural plasticity (Doherty and Walsh, 1991; Goridis and Brunet, 1992). Polysialylation increases the NCAM intrinsic neurite outgrowth-promoting activity (Doherty *et al.*, 1990, 1991; Doherty and Walsh, 1991) and drastically decreases the adhesive properties (Edelman, 1994; Yang *et al.*, 1994). Recent studies demonstrate that PSA is an important substrate for neural cell migration (Rousselot *et al.*, 1995; Hu *et al.*, 1996), axonal growth and path finding (Tang *et al.*, 1994), synaptogenesis (Miller *et al.*, 1994) and synaptic functions associated with learning and memory (Doyle *et al.*, 1992; Muller *et al.*, 1994; Regan and Fox, 1995). The phenotype of NCAM knock-out mice, which includes a small olfactory bulb and deficits in spatial learning (Tomasiewicz *et al.*, 1993; Cremer *et al.*, 1994), seems to be chiefly due to the loss of PSA (Hu *et al.*, 1996). Specific degradation of PSA by *in vivo* application of the PSA-specific endoneuraminidaseNE (endoNE) (Gerardy-Schahn *et al.*, 1995) partially duplicates the mutant phenotype (Ono *et al.*, 1994) and results in impaired learning and long-term potentiation in adult rats (Becker *et al.*, 1996).

Of clinical relevance are findings that demonstrate decreased PSA levels in the hippocampus of patients affected by schizophrenia (Barbeau *et al.*, 1995) and the re-expression of PSA during neural regeneration (Daniloff *et al.*, 1986; Martini *et al.*, 1994) and in neuroendocrine and lymphoblastoid tumours (Komminoth *et al.*, 1991; Metzman *et al.*, 1991; Kern *et al.*, 1992; Takamatsu *et al.*, 1994). In these tumours, PSA has been shown to modulate the malignant potential (Scheidegger *et al.*, 1994).

Studies aimed at elucidating the biosynthesis of PSA have been promoted by the cloning of the polysialyltransferase (Eckhardt *et al.*, 1995; Nakayama *et al.*, 1995; Yoshida *et al.*, 1995). The name polysialyltransferase-1 (PST-1) given to the hamster enzyme (Eckhardt *et al.*, 1995) takes into account the fact that the number of potential polysialyltransferases has not been defined yet. Meanwhile it seems obvious that sialyltransferase X (STX) (Livingston and Paulson, 1993; Kitagawa and Paulson, 1994) possesses similar enzymatic functions and provides a second α2,8-polysialyltransferase (Kojima *et al.*, 1995a,b; Scheidegger *et al.*, 1995). However, the substrate recognized and modified by STX and the catalytic mechanism of this enzyme have not been defined. In contrast, we have shown recently that PST-1 is the only factor required for the polysialylation of NCAM (Mühlenhoff *et al.*, 1996).

The present study concentrates on the catalytic mechanism of PST-1. CHO glycosylation mutants were used to isolate incompletely glycosylated forms of PST-1, which were analysed for their molecular and catalytic features. The findings presented show that PST-1 is a polysialylated enzyme. The polysialylation proceeds via an autocatalytic process and seems to be a prerequisite for PST-1 activity.

Results

Recombinant protein A-tagged PST-1 carries PSA

In analogy to previously described strategies, the eukaryotic expression vector pPROTA (Sanchez-Lopez *et al.*,

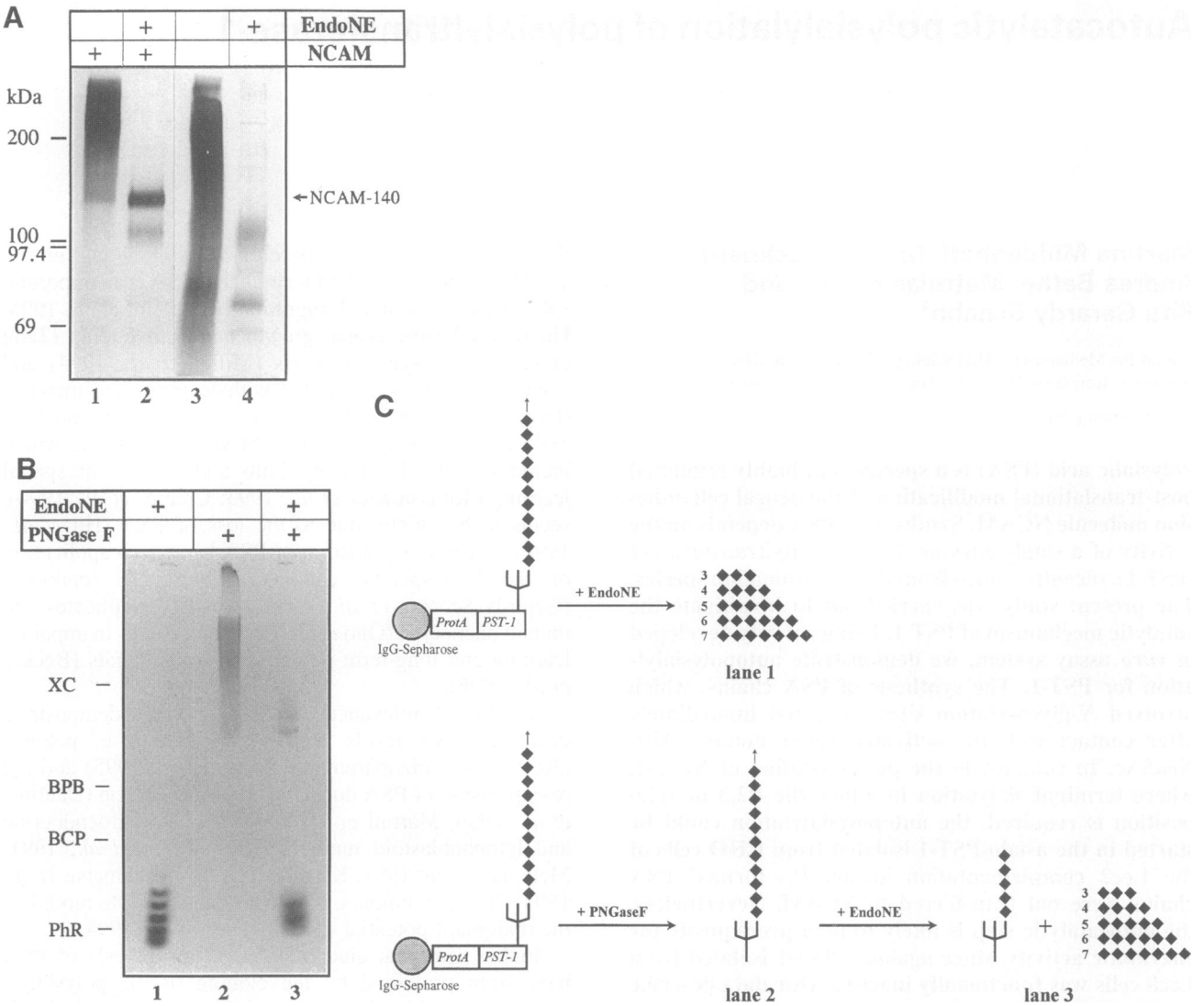

Fig. 1. (**A**) *In vitro* polysialylation of NCAM. PSA-negative human NCAM-140 was immunoprecipitated from COS-hN-6 cells with anti-NCAM antibody 123C3. Immunoprecipitates were incubated for 2 h with CMP-[^{14}C]Neu5Ac and soluble protein A–PST-1 fusion protein from 2A10 cells. Reaction products were analysed before (lane 1) and after treatment with endoNE (lane 2) by 7% SDS–PAGE and autoradiography. In a control experiment, IgG–Sepharose was incubated in the absence of NCAM with soluble protein A–PST-1, and products were analysed before (lane 3) and after endoNE digest (lane 4) as described above. NCAM-140 denotes the molecular weight of NCAM after removal of PSA by endoNE digest. (**B**) Autoradiography of glycosidase-split products generated from autopolysialylated PST-1. ProtA–PST-1 was immunoadsorbed to IgG–Sepharose and incubated for 2 h in the presence of CMP-[^{14}C]Neu5Ac. Parallel samples were treated with endoNE (lane 1), PNGaseF (lane 2) or both enzymes (lane 3). Released sugars were separated on 25% PAGE and visualized by autoradiography. (**C**) The experimental procedure used in (B).

1988) was used to produce a soluble protein A–PST-1 fusion protein (protA–PST-1). In a first experiment, protA–PST-1 was used for *in vitro* polysialylation of immunoprecipitated human NCAM-140. Both protA–PST-1 and human NCAM were expressed in CHO cells of the 2A10 complementation group, which are PSA-negative due to a defect in the PST-1 gene (Eckhardt *et al.*, 1995). The reaction was started by addition of CMP-[^{14}C]Neu5Ac and reaction products were analysed by SDS–PAGE and autoradiography. PSA synthesis was indicated by the appearence of a radioactively labelled high molecular weight smear (Figure 1A, lane 1). Removal of PSA with the PSA-specific endoNE displayed two major bands (lane 2). While the signal at ~140 kDa was NCAM, the nature of the ~100 kDa protein was unknown. Surprisingly, a control experiment carried out in the absence of NCAM, but otherwise identical conditions, also resulted in a broad radioactive smear, which turned into four discrete bands between ~100 and ~70 kDa after digestion with endoNE (lane 4). The ~100 kDa band generated in this sample exactly co-migrated with the band detectable in lane 2. The bands of lower molecular weight detected in lane 4 were also visible in lane 2 but with much weaker intensity. Additional controls, carried out with the supernatant of pPROTA-transfected 2A10 cells in the presence or absence of NCAM, gave no radioactive signals (data not shown), a fact which suggests that the polysialylated material shown in lane 3 is protA–PST-1.

To confirm this point and to investigate how PSA is attached to the protein backbone of PST-1, recombinant protA–PST-1 was isolated on IgG–Sepharose and allowed to undergo autopolysialylation in the presence of CMP-[^{14}C]Neu5Ac. Subsequently, parallel samples were treated with endoNE, peptide-N-glycosidase F (PNGaseF) or both,

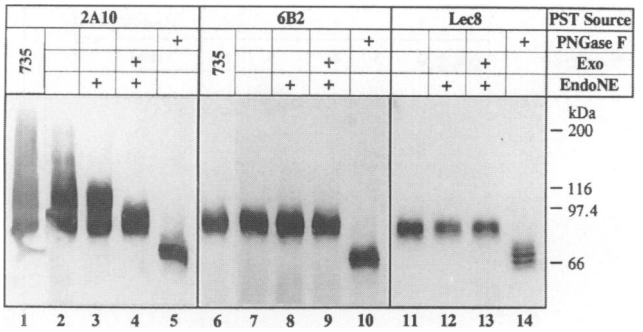

Fig. 2. Western blot analysis of protA–PST-1 isolated from glycosylation-defective CHO mutants. After expression in 2A10, 6B2 and Lec8 cells, respectively, soluble protA–PST-1 was adsorbed to IgG–Sepharose and aliquots were incubated with endoNE, exosialidase from *V.cholerae* or PNGaseF as indicated. The Western blot of an 8% SDS gel was developed with mouse IgG, except for lanes 1 and 6, which were developed with anti-PSA mAb 735.

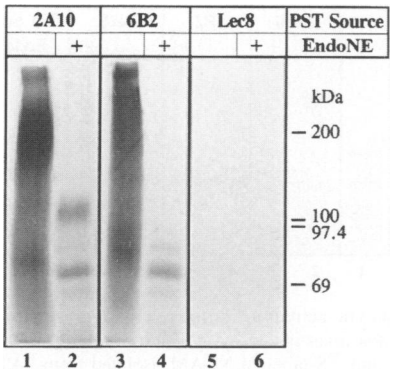

Fig. 3. Autopolysialylation of differentially glycosylated forms of PST-1. ProtA–PST-1 expressed in 2A10, 6B2 and Lec8 cells, respectively, was immunoadsorbed to IgG–Sepharose and incubated with CMP-[^{14}C]Neu5Ac for 2 h. Reaction products were analysed before (lanes 1, 3 and 5) and after treatment with endoNE (lanes 2, 4 and 6) by 7% SDS–PAGE and autoradiography. PSA appeared with the enzymes isolated from 2A10 (lane 1) and 6B2 (lane 3), but not with the agalacto-PST-1 isolated from Lec8 cells (lanes 5 and 6). Specific removal of PSA displayed four bands of ~100, 80, 70 and 64 kDa in the case of 2A10-derived protA–PST-1 (lane 2), but only the three bands of lower molecular weight in the case of 6B2-derived protA–PST-1.

as shown schematically in Figure 1C. After resolution in a high percentage polyacrylamide gel (Pelkonen *et al.*, 1988) and visualization by autoradiography, the products of the endoNE digest migrated as five bands representing oligomeric α2,8-liked *N*-acetylneuraminic acid of 3–7 residues (Fig. 1B, lane 1). *N*-glycan structures liberated from the protein by PNGaseF were radioactive (lane 2) and sensitive to endoNE treatment (lane 3). In summary, these data show PST-1 to be a polysialylated protein. Polysialylation proceeds in an autocatalytic reaction and involves *N*-glycan structures.

Active PST-1 performs autopolysialylation

In order to define the structure of autocatalytically modified *N*-glycans in more detail, the protein A-fused PST-1 was expressed in three different cell lines. A maturely glycosylated enzyme was isolated from transiently transfected CHO cells of the complementation group 2A10 (Eckhardt *et al.*, 1995). CHO 6B2 cells (Eckhardt *et al.*, 1996) belonging to the Lec2 complementation group (Deutscher *et al.*, 1984; Stanley, 1989) were used to isolate an asialo-enzyme and Lec8 cells (Deutscher and Hirschberg, 1986) were used to isolate an agalacto-enzyme. Figure 2 shows a Western blot of the differentially glycosylated enzymes, developed with mouse IgG. When purified from the supernatant of transfected 2A10 cells, PST-1 carried PSA (lanes 1 and 2). Staining of the PSA smear could be enhanced with the PSA-specific mAb 735 (lane 1). EndoNE digestion abolished the microheterogeneous smear but the protein still migrated as a broad band ranging from 87 to 120 kDa (lane 3). Exosialidase treatment further reduced the molecular mass (lane 4) and PNGaseF generated a band of ~70 kDa (lane 5), which correlates well with the calculated molecular weight of protA–PST-1 (67 kDa). The asialo-protein migrated at ~90 kDa (lanes 6 and 7) and the agalacto-form at ~84 kDa (lane 11). While neither endoNE (lanes 8 and 12) nor exosialidase (lanes 9 and 13) were able to reduce the molecular mass, removal of *N*-glycans by PNGaseF digestion again decreased the molecular mass to ~70 kDa (lanes 10 and 14). Moreover, bands of 74, 70 and 66 kDa could be discriminated (lanes 5, 10 and 14), which are the result of an incomplete PNGaseF digest and therefore indicate that at least four of the five potential *N*-glycosylation sites identified in the primary sequence of PST-1 are used.

Autopolysialylation seems to be a prerequisite for catalytic activity. To correlate enzyme glycosylation and enzyme activity, the activities of differentially glycosylated forms of PST-1 were measured. For investigation of the autopolysialylation, differentially glycosylated enzymes were purified on IgG–Sepharose and incubated in the presence of CMP-[^{14}C]Neu5Ac. Figure 3 shows an autoradiograph of the reaction products. Whereas the sialylated and the asialo-form of protA–PST-1 were able to perform autopolysialylation (Figure 3, lanes 1–4), the agalacto-protA–PST-1 was not (lanes 5 and 6). After endoNE digestion, the ~100 kDa signal described in Figure 1 could only be detected in the 2A10-derived enzyme (lane 2). Bands of ~80, ~70 and ~64 kDa were visible in both preparations (lanes 2 and 3). These results allow the conclusion that the ~100 kDa signal, obtained after endoNE digest, represents the completely sialylated enzyme. Bands in the lower molecular weight range are likely to correspond to incompletely glycosylated forms of protA–PST-1. More importantly, this experiment suggests a novel mechanism of glycosylation: the transfer of α2,8-linked PSA to galactose. In contrast to the polysialylation of NCAM, where α2,3- or α2,6-sialylated oligosaccharide cores are required (Mühlenhoff *et al.*, 1996), autopolysialylation of PST-1 could be started in the absence of terminally sialylated core structures.

In order to test the PST-1 activity with the physiological acceptor NCAM, the reaction conditions were modified. Metabolically labelled NCAM ([^{35}S]NCAM), which was immunoprecipitated from 2A10 cells (Figure 4, lane 1), was used in combination with unlabelled CMP-Neu5Ac to ensure clear identification of the modified exogenous acceptor NCAM by autoradiography. PST-1 isolated from 2A10 and 6B2 cells was active and catalysed the polysialylation of NCAM (lanes 2 and 4). The enzyme isolated from Lec 8 cells again had no catalytic activity (lanes 6 and 7). After endoNE digestion, the PSA smear had

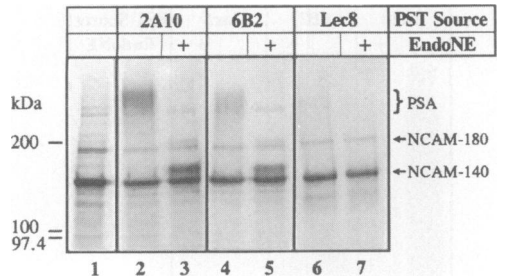

Fig. 4. The catalytic activity of differentially glycosylated protA–PST-1 was investigated by incubation for 2 h with unlabelled CMP-Neu5Ac and ^{35}S-labelled NCAM, isolated from 2A10 cells, which express NCAM-140 and -180 (lane 1). PST-1 activity is indicated by the appearance of the endoNE-sensitive PSA smear. If the reaction was carried out with PST-1 isolated from 2A10 cells (lanes 2 and 3) or from 6B2 cells (lanes 4 and 5), both NCAM isoforms were polysialylated. In contrast, no enzymatic activity was observed with the enzyme isolated from Lec8 cells (lanes 6 and 7). Due to the fact that endoNE does not remove the most proximal α2,8-linked sialic acids of a PSA chain (Finne and Mäkelä, 1985), NCAM bands generated by endoNE digestion migrate slower than the PSA-negative forms of NCAM. Therefore, double bands at ~140 and ~180 kDa (lanes 3 and 5) could be seen after endoNE treatment. NCAM-140 and NCAM-180 denote the molecular weights of the PSA-negative isoforms. PSA depicts the molecular weight area of polysialylated [^{35}S]NCAM.

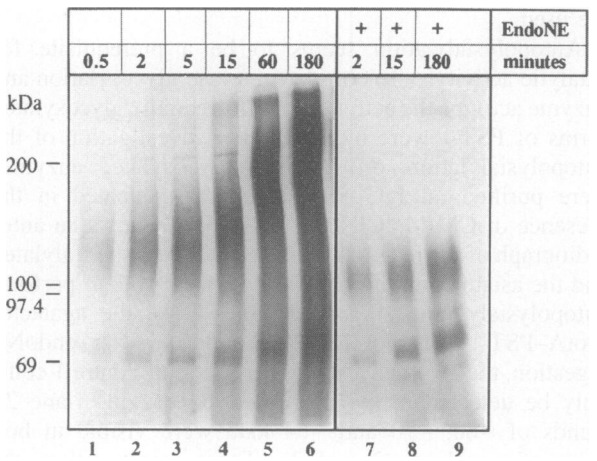

Fig. 5. The kinetics of the autocatalytic polysialylation were determined after addition of CMP-[^{14}C]Neu5Ac to protA–PST-1, adsorbed to IgG–Sepharose. Samples were removed as indicated and analysed by 7% SDS–PAGE and autoradiography. Already after 30 s (lane 1) PST-1 is radioactively labelled and migrates as a smeared band with an average M_r of 150 kDa. The average molecular weight of protA–PST-1 continuously increases with time (lanes 2–6). EndoNE digests were performed with parallel samples after 2 min (lane 7), 10 min (lane 8) and 3 h (lane 9).

disappeared and double bands at ~140 and ~180 kDa became visible (lanes 3 and 5). Since endoNE does not remove the five most proximal sialic acids of a PSA chain (Finne and Mäkelä, 1985), the molecular mass of the polysialylated NCAM is higher after endoNE digest than that of PSA-negative NCAM. Therefore, double bands at 140 and 180 kDa indicate that both NCAM isoforms used in the assay were substrates for *in vitro* polysialylation.

Kinetics of the autocatalytic polysialylation

Figure 5 illustrates the kinetics of the autopolysialylation. After expression in 2A10 cells, protA–PST-1 was adsorbed to IgG–Sepharose and incubated with CMP-[^{14}C]Neu5Ac

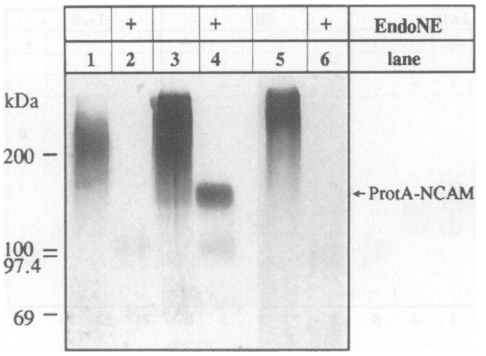

Fig. 6. To investigate a potential transfer of autocatalytically synthesized PSA chains from PST-1 to NCAM, protA–PST-1 was isolated on IgG–Sepharose and pre-polysialylated in the presence of CMP-[^{14}C]Neu5Ac for 2 h. After removal of the unbound radioactivity, PSA-negative protA–NCAM was added to the incubation mixture, alone (lane 1) or together with the activated donor sugar (lanes 3 and 5). The radioactive smear displayed in lanes 1, 3 and 5 indicates PSA synthesis. After specific removal of the PSA smear by endoNE digestion, a radioactive NCAM band could only be detected if CMP-[^{14}C]Neu5Ac was present in the reaction mixture (lane 4). In the absence of the activated sugar donor (lane 2) and in the presence of the unlabelled CMP-Neu5Ac (lane 6), endoNE displayed no signal other than protA–PST-1 at ~100 kDa.

for up to 3 h. Aliquots were removed as indicated and analysed by SDS–PAGE and autoradiography. Under these experimental conditions, the autopolysialylation was not saturable over a period of 3 h. After 30 s, the polysialylated enzyme exhibited an average molecular weight of ~150 kDa (lane 1). The molecular weight and signal intensity increased over time (lanes 2–6). The specificity of the reaction products was confirmed by endoNE digestion after 2, 15 and 180 min (lanes 7–9).

Autocatalytically synthesized PSA chains were not transferred to the acceptor NCAM

The data presented above suggested autopolysialylation to be the first step in the polysialylation of NCAM. Therefore, the next question was whether pre-assembled PSA chains could be transferred from PST-1 to NCAM. Studies carried out to optimize the reaction conditions had shown that the proximity and correct spacing of the interacting partners NCAM and PST-1 are important for optimal polysialylation. Using protA–PST-1 and a soluble NCAM–protein A fusion protein (protA–NCAM), it was possible to adsorb both PST-1 and NCAM to IgG–Sepharose. In the first step, protA–PST-1 derived from 2A10 cells was autopolysialylated for 2 h in the presence of CMP-[^{14}C]Neu5Ac. Free radioactivity was removed by washing and 1 μg of protA–NCAM isolated from transfected 2A10 cells was added to investigate a potential transfer of pre-assembled PSA chains. The reaction was stopped after 2 h and the products were analysed, before and after endoNE digestion, by SDS–PAGE and autoradiography (Figure 6). PSA synthesis was indicated by the appearance of the radioactive smear shown in lane 1. However, removal of PSA by endoNE (lane 2) revealed a single band at ~100 kDa, which represents protA–PST-1. NCAM was not detectable and, therefore, no transfer of radioactively labelled pre-assembled PSA chains could be observed. Nevertheless, the pre-polysialylated enzyme was active. A control experiment, where pre-polysialylated

enzyme was incubated with protA–NCAM in the presence of CMP-[^{14}C]Neu5Ac, resulted in polysialylation of NCAM (lane 3). In this case, a radioactive band of ~150 kDa became visible after endoNE digestion (lane 4). Proceeding from this observation, we tested whether a potential transfer of pre-formed PSA chains could be dependent on the presence of the activated donor sugar. The experiment described above was repeated by simultaneous addition of protA–NCAM and unlabelled CMP-Neu5Ac (lane 5). Again, a transfer of the radioactive label to the NCAM molecule was not detectable (lane 6). Although these data do not exclude the possibility that the enzymatic activity, which finally leads to autopolysialylation, is involved in NCAM polysialylation, they do not support the hypothesis that pre-formed PSA chains are transferred *en bloc* to NCAM.

Discussion

This study addresses issues concerning the catalytic mechanism of PST-1. By means of *in vitro* studies with recombinant protein A-fused PST-1, we unambiguously demonstrate that PST-1 is a polysialylated protein. Polysialylation proceeds via an autocatalytic mechanism on *N*-glycosidic core structures. Surprisingly, the autopolysialylation could be started in the asialo-PST-1 isolated from the CHO cell mutant 6B2. This is in contrast to the polysialylation of NCAM, catalysed by PST-1, which requires the presence of α2,3- or α2,6-sialylated core glycans (Mühlenhoff *et al.*, 1996). Furthermore, the transfer of α2,8-linked PSA chains to β1,4-linked galactose suggests an as yet unknown type of glycosidic linkage. Initial attempts to restore mature core structures in the asialo-PST-1 by use of α2,3- and α2,6-sialyltransferases were accompanied by a reduction in the degree of autopolysialylation (data not shown), confirming the presence of a new unusual glycosidic linkage. It must, however, be noted that this type of reconstitution experiment was largely hampered by the parallel autopolysialylation.

Autocatalytic polysialylation was detectable already after 30 s, indicating that the reaction started immediately after contact with the substrate CMP-Neu5Ac. The molecular mass of the self-modifying enzyme continuously increased for hours and apparently was not saturable under the *in vitro* conditions used. In the presence of NCAM, the kinetics of the autocatalytic polysialylation seem to be altered. While in the absence of NCAM the incompletely glycosylated forms of PST-1 also became modified, only the major band of ~100 kDa was detectable after endoNE digest if NCAM was present (see Figures 1 and 5).

In pulse labelling experiments, Alcaraz and Goridis (1991) could not detect intermediates on the way to completely polysialylated NCAM and, therefore, suggested an *en bloc* transfer of pre-assembled PSA chains to the nascent NCAM molecule. The experiments presented in this study could not demonstrate the transfer of pre-assembled PSA chains from PST-1 to NCAM, although the enzyme in principle was active. Simultaneous addition of protein A-fused NCAM and CMP-[^{14}C]Neu5Ac to the autocatalytically modified PST-1 resulted in polysialylation of NCAM (Figure 6, lanes 3 and 4). Elucidation of the catalytic mechanism of PST-1 needs further experimental efforts. However, on the basis of our data, we may speculate that PST-1 is an enzyme with two catalytic activities. Whether the two activities are related or independent remains to be shown. Two results suggest autopolysialylation to be a prerequisite of PST-1 activity: (i) the agalacto form of PST-1 showed no autocatalytic activity and was not able to polysialylate NCAM; and (ii) the autopolysialylated form of PST-1 was detectable under all experimental conditions, even in the presence of NCAM.

In performing autocatalytic modification by α2,8-polysialylation, PST-1 provides a further example of those enzymes which are self-maturating via polymerization reactions. This extreme form of post-translational modification has been described recently for the poly(ADP-ribose)polymerase, PARP (Lindahl *et al.*, 1995), and for glycogenin (Alonso *et al.*, 1995). PARP is an abundant nuclear protein involved in the repair of DNA strand interruptions. PARP binds to DNA strand breaks and immediately undergoes automodification with synthesis of long branched polymers of highly negatively charged poly(ADP)ribose (Lindahl *et al.*, 1995). Glycogenin is a self-glucosylating protein, which performs the first steps of the glycogen synthesis (Alonso *et al.*, 1995). While the latter enzymes transfer the first sugar to an amino acid residue in the peptide backbone (glutamic acid in the case of PARP, threonine in the case of glycogenin), autopolysialyation of PST-1 involves *N*-glycans. A common feature of the three enzymes is the fact that the autocatalytic mechanisms are not understood and, in the case of PST-1 and PARP, the functional importance awaits further clarification.

In conclusion, our data provide a new basis for studies aimed at elucidating PST-1 catalysis and should help to sort out experimental differences obtained in PST-1 acceptor studies in different laboratories (Easton *et al.*, 1995; Yoshida *et al.*, 1995; Nakayama and Fukuda, 1996). All assay systems described so far summarize the appearance of PSA instead of identifying the PSA carrier molecule and, therefore, bear a high risk of producing false-positive results. This study demonstrates that the identification of the PSA carrier is essential.

Materials and methods

Materials

CMP-Neu5Ac was purchased from Sigma, CMP-[^{14}C]Neu5Ac (293 μCi/mmol, 1 mCi = 37 MBq) and [^{35}S]methionine (sp. act. >1000 Ci/mmol) from Amersham. EndoNE was purified from the *Escherichia coli* K1 bacteriophage, PK1E, as described (Gerardy-Schahn *et al.*, 1995). The monoclonal antibody (mAb) H28 (rat IgG2a), directed against all isoforms of mouse NCAM (Hirn *et al.*, 1981), and mAb 123C3 (IgG1), reactive with all isoforms of human NCAM (Moolenaar *et al.*, 1990; Gerardy-Schahn *et al.*, 1994), were kindly provided by Dr C.Goridis (CNRS, Marseille, France) and Dr R.Michalides (The Netherlands Cancer Institute, Amsterdam). Both, mAb H28 and mAb 123C3, were used after affinity purification on protein G–Sepharose (Pharmacia). mAb 735 (IgG2a) directed against polysialic acid (Frosch *et al.*, 1985) was used after purification on protein A–Sepharose (Pharmacia).

Cell lines

The 2A10 cells belong to a CHO complementation group, which is PSA-negative due to a defect in the PST-1 gene. This cell line has been isolated previously in our laboratory and shown to express mature complex core structures (Eckhardt *et al.*, 1995). Isolated after chemical mutagenesis of wild-type CHO cells, the clone 6B2 was identified as a member of the Lec2 complementation group (Stanley, 1989). Cells of the Lec2 group exhibit a defect in the Golgi CMP-sialic acid transport

system and therefore express asialo-glycan structures (Deutscher *et al.*, 1984; Eckhardt *et al.*, 1996). Lec8 cells were obtained from the American Type Culture Collection (ATCC CRL 1737). Due to a defect in the Golgi UDP–galactose transporter, Lec8 cells express agalacto-glycoproteins and glycolipids (Deutscher *et al.*, 1984; Deutscher and Hirschberg, 1986). CHO cell lines were grown in α-MEM (Gibco) supplemented with 10% fetal calf serum (FCS) and 10 mM sodium pyruvate. COS-hN-6 designates COS-M6 cells stably transfected with human NCAM-140. COS-hN-6 cells previously established in our laboratory (Eckhardt *et al.*, 1995) were cultured in DMEM (Gibco) containing 5% FCS. All media were supplemented with 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 U/ml).

Transfection and expression of soluble protein A-fused proteins

The vector pPROTA (Sanchez-Lopez *et al.*, 1988) (generously provided by Dr R.Breathnach, INSERM, Nantes, France) was used for the construction of soluble protein A-fused proteins. The oligonuclotide primers ME12 5′-CCAGAACTGAGGAGCAC-3′ and ME22 5′-TTATTGCTTCATGCACTTCCC-3′ were used to amplify the hamster PST-1 sequence (Eckhardt *et al.*, 1995) without the transmembrane domain, and the primers MM13 5′-TGCAGGTGGATATTGTTCCC-3′ and MM14 5′-TCAGVVTGAGGTGGGGCTGC-3′ to amplify the extracellular domain of human NCAM by PCR. The vector was linearized with *Eco*RI, filled in with Klenow polymerase, and the PCR fragments were inserted by blunt end ligation. The resulting plasmids pPROTA-PST-1 and pPROTA-NCAM contain the transin signal sequence and the IgG binding domain of *Staphylococcus aureus* protein A in front of the truncated proteins. Correct orientation of the cloned PCR fragments was confirmed by sequencing, and proteins were expressed by transient transfection in 2A10, 6B2 or Lec8 cells with lipofectamine (Gibco) as described previously (Mühlenhoff *et al.*, 1996). At 72 h after transfection, cell supernatants were collected and used for isolation of protein A fusion proteins. For the isolation of protA–NCAM, the supernatants were passed though IgG–Sepharose columns. After washing with five column volumes of phosphate-buffered saline (PBS; 10 mM sodium phosphate pH 7.4, 150 mM NaCl), the protein A fusion protein was eluted with glycine buffer (100 mM glycine, pH 2.7) and dialysed overnight against PBS. ProtA–PST-1 was isolated by addition of 10 µl (volume of wet beads) of IgG–Sepharose (Pharmacia) per ml of supernatant. The extraction was carried out for 2 h at 4°C with gentle rocking. Beads were washed twice with 1 ml of 50 mM Tris–HCl (pH 8.0), 500 mM NaCl and three times with 1 ml of 10 mM sodium cacodylate (pH 6.5), 10 mM $MnCl_2$, resuspended in the same buffer and used as an enzyme source. Alternatively, the cell culture supernatant was harvested, concentrated by factor 10 using Centricon-30 filters (Amicon), dialysed against 10 mM sodium cacodylate (pH 6.5), 10 mM $MnCl_2$, and used as an enzyme source.

In vivo labelling

CHO-2A10 cells (2×10^6) were grown overnight on culture dishes (6 cm diameter), washed three times with methionine-free MEM (Gibco) and incubated for 1 h in 1 ml of the same medium, containing 10% FCS, dialysed against PBS. Subsequently, 100 mCi of [^{35}S]methionine (Amersham) was added and the cells were incubated for another 6 h. Labelling was stopped by washing twice with 1 ml of PBS, and cells were lysed on the plate with 1 ml of ice-cold lysis buffer I [50 mM Tris–HCl pH 8.0, 1% NP-40, 2% CHAPS, 10 mM EDTA, 1 mM $MgCl_2$, 10 U/ml aprotinin and 1 mM phenylmethylsulphonyl fluoride (PMSF)]. Cell lysates were used for immunoprecipitation as described below.

Immunoprecipitation of NCAM

A total of 10^7 cells (or 2×10^6 cells in the case of *in vivo* labelled cells) were lysed in 1 ml of lysis buffer II (50 mM Tris–HCl pH 8.0, 1% NP-40, 1 mM $MgCl_2$, 10 U/ml aprotinin and 1 mM PMSF). After centrifugation at 14 550 *g* for 5 min, aliquots of the supernatant were immunoprecipitated for 2 h at 4°C. Human NCAM from COS-hN-6 cells was precipitated with the mAb 123C3 covalently bound to protein A–Sepharose (Pharmacia). NCAM from CHO cells was precipitated with the mAb H28 covalently bound to protein G–Sepharose (Pharmacia). Immunoprecipitates were washed twice with 1 ml of 50 mM Tris–HCl (pH 8.0), 0.5% NP-40, 500 mM NaCl and twice with the same buffer without NaCl. Final washing was carried out with 1 ml of 10 mM sodium cacodylate (pH 6.5), 10 mM $MnCl_2$, and beads were resuspended in the same buffer.

Enzymatic treatment of immunoprecipitates

All incubation steps were performed with 10 µl of beads (protA–PST-1 or protA–NCAM adsorbed to IgG–Sepharose, or immunoprecipitated NCAM, respectively) in a final volume of 50 µl at 37°C in a Thermomixer (Eppendorf) at 1400 r.p.m. Before the enzymatic treatments, immunoprecipitates were washed twice with 1 ml of the subsequent reaction buffer. For polysialylation with ^{14}C-labelled Neu5Ac, immunoprecipitates were incubated for 2 h with 125 nCi CMP-[^{14}C]Neu5Ac in 10 mM sodium cacodylate (pH 6.5), 10 mM $MnCl_2$. Polysialylation of ^{35}S-labelled NCAM was carried out under the same conditions with CMP-Neu5Ac (Sigma) at a final concentration of 1.6 mM. For kinetic studies of the autopolysialylation, the polymerization reaction was stopped by adding 1 ml of 20 mM Tris–HCl (pH 8.0), 10 mM EDTA. Beads were centrifuged (14 500 *g* for 30 s) and covered immediately with 30 µl of Laemmli sample solution. For specific degradation of PSA, immunoprecipitates were digested in PBS with 100 ng of endoNE for 1 h. Exoneuraminidase treatment was done for 1 h in 50 mM sodium acetate pH 5.5, 4 mM $CaCl_2$ with 3 mU of neuraminidase from *Vibrio cholerae* (Boehringer Mannheim), and removal of *N*-linked glycan structures was performed for 4 h in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA with 1 U of PNGaseF (Boehringer Mannheim).

Analysis of oligo- and polysaccharides by high percentage PAGE

Carbohydrates released by the different neuraminidases and PNGaseF, respectively, were separated from immunoprecipitated proteins by centrifugation (14 550 *g* for 2 min). Forty µl of the resulting supernatant were analysed on 25% polyacrylamide gels as described (Pelkonen *et al.*, 1988). Electophoresis was performed at 4°C, 400 V for 4 h, using 14 cm gels (0.8 mm thick).

SDS–PAGE and Western blotting

After washing with 1 ml of PBS and addition of Laemmli sample buffer, containing 5% β-mercaptoethanol, immunoprecipitates were analysed by SDS–PAGE. Samples were heated for 20 min at 60°C, centrifuged at 14 550 *g* for 2 min and loaded on 8 or 7% SDS–polyacrylamide gels. After electrophoresis, proteins were blotted onto nitrocellulose membranes and the membranes were blocked for 1 h by incubation with blocking buffer (2% non-fat dry milk in 20 mM Tris–HCl pH 8.0, 150 mM NaCl) for 1 h. Incubations with mAb 735 and mouse IgG (Pierce), both used at a concentration of 5 µg/ml in blocking buffer, were performed for 1 h. After three washes in Tris-buffered saline (TBS; 20 mM Tris–HCl pH 8.0, 150 mM NaCl), blots were incubated with alkaline phosphatase-conjugated anti-mouse IgG $F(ab)_2$ fragments (Dianova) in a 1:500 dilution for 30 min. The reaction was stopped by washing twice in TBS and once in TBS-A (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Blots were developed by incubation with 150 µg/ml 5-bromo-4-chloro-3-indoyl phosphate and 300 µg/ml nitro-blue tetrazolium in TBS-A.

Autoradiography

SDS gels and high percentage acrylamide gels were vacuum dried immediately after electrophoresis and exposed to Hyperfilm-MP (Amersham).

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