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More than silk and honey – or, can insect cells serve in the production of therapeutic glycoproteins?

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Why deal with protein glycosylation in insect cells?

More than one million insect species are known today and many of them convey profound effects on our life. They pollinate plants, they supply us with honey, they fill Mediterranean nights with their music and they serve as protein-rich food – even if not for all of us. On the other hand, insects destroy our harvests, mosquitos harass us at night, bees, wasps and hornets hurt us with their stings and by doing so may even kill us, either with a little help from ourselves in the case of bee venom allergy or from tiny parasites as in the case of malaria etc. These points amply justify research on the biochemistry of insects including protein synthesis and modification.

An especially strong motivation to study glycosylation and other post-translational modifications in insect cells is that they can serve for the production of recombinant proteins. Currently, the baculovirus/lepidopteran insect cell system is widely used for the production of laboratory scale amounts of recombinant proteins for scientific purposes. Improved cell culture conditions [1] or alternative insect cell expression systems [2] may render insect cells even more attractive in terms of volumetric yield and simplicity of the cloning process. Insects are phylogenetically very distant from humans and their cells can be grown on serum-free medium. Thus, there is minimal danger of uncontrolled transmittance of human-pathogenic DNA or protein elements such as viruses or prions – provided the insect cells are grown in the absence of fetal serum.

Unlike bacterial expression systems, insect cells usually produce soluble, bio-active and post-translationally modified recombinant proteins. N-glycosylation, O-glycosylation and even phosphorylation [3] take place and the odds are high that they occur at the correct positions. The recombinant proteins therefore will usually fulfil physicochemical requirements of solubility and stability. However, for therapeutical use the requirements are more stringent. Glycosylation of a recombinant protein will always to some degree deviate from that of the natural protein. The point is to guarantee that such deviations do not have any harmful effects. Clearly, glycan structures against which antibodies exist in the serum or which elicit an immune-response should be absent. For example, most animal cell lines furnish glycoproteins with the Galα1-3Galβl-4GlcNAc-epitope, against which humans have high levels of antibody in their serum [4]. The widespread use of CHO-cells arises, not at least, from the fact that this cell line, like humans and apes, is devoid of α1,3-galactosyl-transferase activity. Terminal GlcNAc-residues at the non-reducing end of N-glycans likewise appear problematic considering their role in rheumatoid arthritis [5]. Fucose in α1,3-linkage to the Asn-bound GlcNAc residue, a structural element found in plant as well as in insect glycoproteins renders the glycan immunogenic [6].

But what about N-glycan structures which are tolerated by the immune system, but do not occur on the natural protein? With only a few exceptions, *eg* human chorionic gonadotropin, the precise structure of N-glycans appears to exhibit little if any direct effect on the biological activity of a glycoprotein. Taken to the extreme: would a recombinant protein with oligomannosidic instead of sialylated bi- or triantennary glycans be acceptable, provided it was pharmacokinetically suitable? While this question has not yet been decided (neither by the legal authorities nor by the scientific community), time should be taken to evaluate the glycosylation potential of the various expression systems such as insect cells, plants and fungi.

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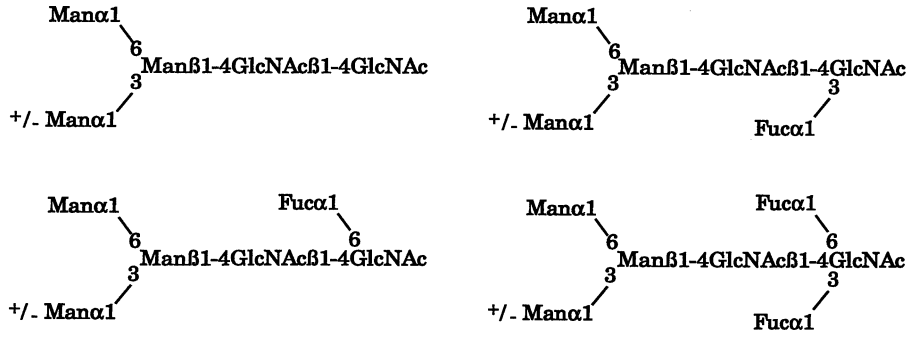

Figure 1. N-glycan with a GlcNAc residue on the 3-arm.

N- and O-glycans found on insect cell-derived, recombinant proteins

In most cases, at least the N-glycosylation of insect cell-derived recombinant proteins has been addressed in some way, *eg* by lectin blotting or endo-*N*-acetylglucosaminidase H digestion. But only a few groups have so far elucidated N- or O-glycan structures by more powerful and reliable methods including HPLC-procedures and NMR. The results which are only summarized here, are presented in more detail in a recent review [8].

Firstly, insect cells do O-glycosylate. However, the O-glycans consist at best of the disaccharide Galβ1-3GalNAc [9]. Galactosylation tends to be rather incomplete so that single O-GalNAc residues can be found. It is not known whether strictly the same O-glycosylation sites are used by insect and mammalian GalNAc-transferase. The N-glycans may belong to the oligomannosidic series (Man5-9GlcNAc2) [10–14]. In addition or sometimes exclusively, truncated N-glycans containing three or only two mannoses and often fucose attached α1,6 to the Asn-bound GlcNAc were found [11, 15, 16]. While natural insect glycoproteins were shown to contain fucose also in α1,3-linkage (Figure 1), the same techniques did not point to the presence of this residue in recombinant proteins ([16] Schwihla H. and Glößl J., unpublished results). Nevertheless, special attention must be paid to this immunogenic structural element.

This picture was further complicated in 1991 by a few reports on sialic acid-containing, complex-type N-glycans in human plasminogen expressed in Sf21 and other insect cell lines [13, 14, 17, 18]. The occurrence of complex N-glycans and the expression of the respective glycosyltransferase was attributed to the baculovirus infection process. Since then, no other group has been able to confirm these findings. On the contrary, studies designed to examine the effect of virus infection on the glycosylation apparatus of insect cells did not show such an influence [19, 20]. It should be noted that for the above mentioned studies [13, 14, 17, 18], rather unusual methods were employed: *eg*, for compositional analysis the samples were not depolymerized by acid hydrolysis as is usual, but instead, were enzymatically cleaved using a clam liver extract. More or less the only criterion for structural identification was high pH anion exchange chromatography/pulsed amperometry which can be a source of error for identification. It is surprising that the usually cumbersome and tedious process of peer reviewing failed in this case to force authors to verify an unexpected result by another, independent method.

In summary insect cells cannot produce sialylated, complex N-glycans. In addition human lysosomal enzymes appear not to be furnished with the mannose-6-phosphate tag in insect cells, but rather with truncated N-glycans ([21], Schwihla H. and Glößl J., unpublished results).

N-glycans of natural insect glycoproteins

The structural analysis of natural insect glycoproteins such as phospholipase A2 from *Apis mellifera*, apolipophorin III from *Locusta migratoria*, and membrane glycoproteins from cultured insect cells [22–24] revealed some other highly interesting features of the insects' glycosylation potential (which again are presented in more detail in ref. [8]). The occurrence of: fucose linked α1-3 to the Asn-bound GlcNAc, hitherto believed to be typical only for plants; GlcNAc on the α1,3-arm of the core-pentasaccharide; GlcNAc on the α1,6-arm of the core-pentasaccharide; 2-aminoethylphosphonate linked to terminal GlcNAc or mannosyl residues; the trisaccharide antenna GalNAcβ1-4(Fucα1-3) GlcNAc.

N-glycan processing in insect cells

The occurrence of Man3GlcNAc2Fuc glycans is not consistent with the classical N-glycan processing pathway established for mammalian cells, since in mammals both the α-mannosidase II and the fucosyltransferase require the presence of GlcNAc-1 which is transferred by GlcNAc-transferase I. In principle, the fucosylated paucimannose structures could be explained by a different specificity of

both the α-mannosidase, removing the two terminal mannosyl residues from the α1,6-branch, and the fucosyltranserase(s) acting on the innermost GlcNAc. However, four recent observations make this hypothesis unlikely. (1) Insect cells do contain appreciable GlcNAc-transferase I activity [19, 25] and structures containing the respective GlcNAc-residue have been found (see above section); (2) The fucosyltransferase(s) of insect cells do(es) not act upon Man3GlcNAc2 [19]; (3) α-mannosidase II from insect cells is also strictly dependent on the presence of GlcNAc-1 [26]; and (4) GlcNAc-2 is transferred to the α1,6-antenna only after the action of GlcNAc-transferase I [19].

The finding of a membrane-bound *β-N*-acetylglucosaminidase with strict specificity for the α1,3-branch suggests that the sequence of trimming and redecoration events is similar to that in mammalian cells. Only at the point when galactosylation would occur in mammalian cells, do insect cells diverge, mostly by removing the GlcNAc residue linked to the α1,3-arm of the core-pentasaccharide with a 'processing *β-N*-acetylglucosaminidase' [27].

Insect cells as expression systems for therapeutic glycoproteins

The general dissimilarity of insect and mammalian protein-glycans and the possible occurence of core-α1,3-fucosylated N-glycans appear to argue against the therapeutic use of insect cell derived glycoproteins because of possible immunological complications. Is this the end of the story of the insect cell expressed glycoprotein therapeutics? Perhaps yes, but not necessarily. It may be possible to obtain cell lines devoid of α1,3-fucosyltransferase activity either by conventional mutation/screening procedures or, more appealingly, by specific inactivation of the respective gene. The latter is presently not feasible because the transferase gene has not yet been found, and because a readily applicable procedure for gene knock-out in cultured cells is not available. But in the near future these problems could be solved. Now we have to consider four options:

(I) Accept the paucimannosidic (not 3-fucosylated) N-glycans. Truncated N-glycans or a mixture of truncated N-glycans and oligomannosidic N-glycans can be an acceptable decoration of a potential therapeutic glycoprotein. At least one therapeutic agent with such N-glycans is in clinical use, namely glucocerebrosidase, for the treatment of Gaucher's disease [28]. Presently, this protein is obtained either from healthy individuals or from mammalian cell cultures and submitted to a series of exoglycosidase digestions to yield Man3GlcNAc2(Fuc) glycans. Alternatively, glucocerebrosidase is expressed in GlcNAc-transferase I deficient CHO cells (Lec1 mutant), containing mainly Man5GlcNAc2 structures. Both mannose-terminated glycoforms are taken up by macrophages via the macrophage mannose-receptor. Insect cells appear to be a perfect expression system for such mannose-terminated glycoprotein.

(II) Construct a cell line which produces true oligomannosidic N-glycans only. This could be achieved by inactivation of the insect cell's GlcNAc-transferase I either by mutation or specific knock-out. Due to the specificity of 3-fucosyltransferase, inactivation of this enzyme would then not be necessary. Generally this Lec1-like approach (GlcNAc-transferase I deficient insect cells) appears to be the one which probably best avoids any complications arising from the presence of N-glycan structures which are non-self for the human immune system. Would such glycoproteins be applicable even if the native form contained complex N-glycans? If the answer is yes, There is a good chance that insect cells will serve as an expression system for glycoprotein therapeutics on a broader scale.

(III) Induce an insect cell line to produce complex N-glycans by cloning of the required glycosyltransferases. Quite a number of transferases have to be stably expressed, their localization and their activity levels must be appropriate and the donor substrates must be provided by the insect cells. Thus, presumably NeuNAc-synthetase would have to be cloned, etc. Taken together, this might well be the most ambitious genetic-engineering effort ever considered.

(IV) *In vitro* modification of the purified glycoprotein by the use of (recombinant) glycosyltransferases. Different starting points for this approach are possible, *eg* Man5GlcNAc2 structures (see II) could be used. Most promising appears to be an approach starting with 3-fucosyltransferase-deficient cells (as mentioned in I). Such cells would yield the partially 6-fucosylated, paucimannosidic structures shown above. The cell's endogenous GlcNAc-transferase I could be used directly to obtain structures with a GlcNAc on the 3-arm (Figure 2).

For that purpose, the action of the insect cells' 'processing *β-N*-acetylglucosaminidase' must be prevented, either by the use of inhibitors, anti-sense RNA or gene inactivation. These insect cell derived glycoproteins containing GlcNAc-Man3GlcNAc2 (Fuc) glycans would constitute a better starting material for enzyme-aided redecoration than glycoproteins from fungal systems which contain only oligomannosidic glycans. However, any advantage in cost-effectiveness of insect over mammalian cells could be out-balanced by the costs of these post-fermentation modifications. The 'glyco-cosmetic' approach holds an advantage not even offered by mammalian cells: maximal freedom in choice of the desired glycosylation pattern.

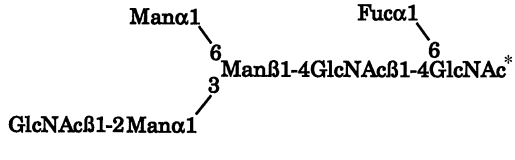

Figure 2. Truncated (or paucimannosidic) N-glycans of insect glycoproteins. (The *a*1,3-mannosyl residues may be absent to some degree). The * immunogenic, *a*1,3-fucosylated structures have so far been detected only in natural insect glycoproteins.

Conclusions

Despite all these considerations, there is a good prospect for the therapeutic use of non-glycosylated and even of glycosylated proteins expressed in insect cells, provided the latter do not have to carry sialylated complex-type N-glycans. This restriction means that insect cells can only be regarded a generally applicable system for the expression of recombinant glycoproteins when the 'glyco-cosmetic' remodelling of their N-glycans has become technically and economically feasible.

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