The Cytoskeletal Protein Talin Is O-Glycosylated*

Jörg Hagemann, Marianne Grob, and Max M. Burger
From the Friedrich Miescher Institut, CH-4002 Basel, Switzerland

(Received for publication, November 26, 1991)

Talin is a large cytoskeletal protein with an apparent molecular mass of 225–235 kDa (Burridge and Connell, 1983), and an actual molecular mass, based on cDNA sequencing, of 270 kDa (Rees et al., 1990). It occurs in many different tissues and tissue culture cells at sites where actin filaments are linked to the cell membrane. Examples are focal contacts, where stress fibers anchor cells to the extracellular matrix by protein kinase C on serine or threonine residues, and by their regulation, however, are obscure. Phosphorylation linked to the cell membrane. Examples are focal contacts, cytoplasmic side of the postjunctional membrane of neuro-muscular junctions (Sealock et al., 1986), and with another intercalated discs of cardiac muscle (Belkin et al., 1986). At the molecular level, talin interacts with the cytoplasmic domain of integrins (Horwitz et al., 1986), and with another cytoskeletal protein, vinculin. The role of these interactions and their regulation, however, are obscure. Phosphorylation by protein kinase C on serine or threonine residues, and by pp60src on tyrosine, has been reported and may play a role in modulating structures containing talin (Turner et al., 1989).

Alternatively, digestion of talin by the Ca2+-dependent protease calpain may lead to the breakdown of focal contacts (Beckerle et al., 1987, but see also Turner et al., 1989).

An unexpected modification of intracellular proteins has been found recently. Despite earlier reports (e.g. Meyer and Burger, 1976), it was generally believed that glycosylated proteins are confined to the extracellular compartment. However, analysis of proteins labeled with UDP-[3H]galactose and galactosyltransferase revealed a large number of both nuclear and cytosolic proteins carrying O-linked GlcNAc residues (Holt and Hart, 1986). Among the few cytosolic proteins identified so far is the cytoskeletal protein 4.1 (Holt et al., 1987a; Inaba and Maede, 1989) which has limited homology with talin (Rees et al., 1990). Here we report that chicken gizzard talin is another cytoskeletal protein carrying GlcNAc moieties and identify the peptide sequences involved. Interestingly, the glycosylation site is not located in the amino-terminal head domain containing the sequences homologous to protein 4.1.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Talin was isolated from frozen chicken gizzard, porcine stomach, and from human platelets as described by O’Halloran et al. (1986). One batch of platelets was activated with 0.2 units/ml of thrombin for 10 min at 37°C and then processed as control platelets. The identity of talin stemming from the three different sources was confirmed by immunoblotting with a monoclonal antibody raised against chicken gizzard talin which recognized all three forms (not shown). Vinculin and α-actinin were obtained from chicken gizzard by the method of Feramisco and Burrige (1980). Vinculin was further purified by hydroxyapatite chromatography (Wilkins and Lin, 1986).

Galactosyltransferase Labeling of Proteins—Talin or the 190-kDa fragment of talin was labeled with [3H]galactose according to the method of Holt et al. (1987a). Briefly, 5 μg of the protein were incubated with 20 milliliters of galactosyltransferase, 10 μCi of UDP-[3H]galactose, 1.25 mM 5’-AMP, 5 mM galactose, 0.5 mM MgCl2, 0.1 mM MnCl2, 0.25% Nonidet P-40, 0.5% apronitin, and 7 mM Hepes-HCl, pH 7.3, in a total volume of 100 μl at 37°C for 30–60 min.

β-Elimination and Endoglycosidase F Treatment of [3H]Galactose-labeled Talin—β-Elimination of the [3H]galactose-labeled sugar moiety attached to the 190-kDa fragment of talin was performed in 0.1 M NaOH and 1 M NaBH4 for 18 h at 37°C. After neutralization with 25% acetic acid, NaBH4 was removed by repeated extraction with methanol. The mixture was chromatographed on a Superose 12 column, and the labeled saccharide which eluted with the included volume was analyzed by thin layer chromatography on Silica Gel 60 plates developed in n-butanol:acetic acid:H2O, 20:10:15. Galβ1-4GlcNAcitol was used as a standard.

Endoglycosidase F treatment was performed according to the method of Holt and Hart (1986). Briefly, 10 μg of acetone-precipitated [3H]galactosylated talin were incubated with 10 units/ml of glycopeptidase F in 150 mM sodium phosphate buffer, 20 mM EDTA, 1% β-mercaptoethanol, pH 8.0, for 20 h at 20°C and analyzed by SDS-PAGE and fluorography.

Purification of the 190-kDa Chymotryptic Fragment—140 μg of purified chicken gizzard talin were digested with 2.8 μg of chymotryp-

* 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.

1 To whom correspondence should be addressed: Friedrich Miescher Institut, P. O. Box 2643, CH-4002 Basel, Switzerland.

1 The abbreviations used are: UDP-galactose, uridine 5’diphosphogalactose; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPCK, 1-tosyl-aminomethyl-2-phenylethyl chloromethyl ketone; TLCK, 1-chloro-4-tosylamido-2-aminomethyl-2-heptanone; HPLC, high performance liquid chromatography.
sin in a solution of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, at 0 °C for 90 min. The reaction was stopped by the addition of 4 mM phenylmethylsulfonyl fluoride, and the pH was adjusted to 6.8. The resulting 190-kDa fragment was separated on a Mono Q HPLC column equilibrated with 20 mM Tris-HCl, 1 mM EDTA, 0.02% sodium azide, pH 6.8. The peptides were eluted with a linear gradient of NaCl (0–400 mM in 20 mM Tris-HCl, 0.02% sodium azide). 0.5-ml fractions were analyzed by SDS-PAGE.

Isolation of Peptides Labeled with [3H]Galactose—After labeling with [3H]galactose, the 190-kDa fragment was separated from contaminating fragments, the galactosyltransferase, and from unreacted UDP-[3H]galactose by chromatography on a Superose 12 column in 150 mM NaCl, 0.1 mM EDTA, 0.1% mercaptoethanol, 20 mM Tris acetate, pH 7.6. Subsequently, the fragment was eluted on a Mono Q ion exchange column and eluted with a 0–500 mM gradient of NaCl. After adjusting the pH to 8.0, 180 pg of the purified fragment was digested twice with 3.6 pg of TPCK-trypsin for 2 h at 37 °C. The resulting mixture was further incubated with 3.6 μg of protease V8 overnight at 37 °C.

The peptides were separated by reverse phase HPLC. A C-18 Vydac column (2.1 × 250 mm) equilibrated with 0.1% trifluoroacetic acid was eluted with a linear gradient of acetonitrile (0–70%) at a flow rate of 0.2 ml/min. Fractions corresponding to peaks of absorption at 214 nm were collected individually, and 10-μl aliquots were counted in a scintillation counter.

Amino Acid Sequencing—Peptides were sequenced on a 470A protein sequenator (Applied Biosystems).

SDS-PAGE, Fluorography, and Blotting—SDS-PAGE was performed as described by Laemmli (1970). For fluorography, the fixed gels were treated with 1 M salicylate, dried, and exposed to Kodak X-Omat AR diagnostic film for 3–7 days. Blotting onto Immobilon polyvinylidene difluoride (Millipore) was done using a semi-dry electroblotter from JKA Biotech, Denmark.

Materials—Galactosyltransferase from human milk, 5'-AMP, and glycopeptidase F were purchased from Boehringer, Mannheim, Federal Republic of Germany. TLCK-chymotrypsin, TPCK-trypsin, V8, apronitin, and thrombin were from Sigma, d(+)-galactose from Fluka, Buchs, Switzerland, and protein A-Sepharose CL 4B was from Pharmacia. UDP-d-[6-3H]galactose (20 Ci/mmol, TRK 513) was from Amersham, United Kingdom. Galβ1–4NActitol was a gift from Dr. B. Ernst, Ciba-Geigy, Basel, Switzerland.

RESULTS

Labeling of Chicken Gizzard Talin by UDP-[3H]Galactose and Galactosyltransferase—In order to demonstrate glycosylation of chicken gizzard talin, the protein was labeled with tritiated UDP-galactose as described under "Experimental Procedures." It is important to realize that the galactosylation reaction was performed in vitro serves as a marker for terminal GlcNAc residues preexisting in vivo. Fig. 1 shows that the label is incorporated into talin, but not into α-actinin and vinculin, two other cytoskeletal proteins. Galactosylation of triton extracts (crude cytoskeletal preparation) results in the labeling of a small number of bands (not shown). Similar observations and detailed studies on the subcellular localization of glycosylated proteins have been published before (Holt et al., 1987a, Holt and Hart, 1986). The label incorporated into talin is released by β-elimination and comigrates with Galβ1–4NActitol (Fig. 2). This indicates that the accepting sugar is GlcNAc O-linked to the protein, a conclusion corroborated by the finding that treatment with endoglycosidase F does not remove the labeled carbohydrate (data not shown). Only 0.06 mol of galactose is incorporated into 1 mol of talin by galactosyl transferase. Therefore, at most 6% of all talin molecules are exhibiting terminal GlcNAc moieties.

Molony et al. (1987) have demonstrated that the Stokes radius of talin changes depending on the ionic strength of the buffer. We investigated the effect of low and high ionic strength buffers on the galactosylation of talin. 0.047 and 0.06 mol of [3H]galactose were maximally incorporated into 1 mol of talin in 10 mM and 150 mM NaCl, respectively. The apparent Km of the reaction was also not affected (20 μM at 10 mM NaCl and 25 μM at 150 mM NaCl).

Localization of the Glycosylation Sites—In order to further characterize the glycosylation of talin, the localization of the galactosylated sites and the sequences adjacent to putative acceptor residues were determined. Talin readily decays during purification and storage (Molony et al., 1987)2 The resulting fragments are a large 190-kDa peptide and a shorter peptide with a molecular mass of about 47 kDa. Similar fragments are generated by the Ca2+-dependent protease calpain and by chymotrypsin (Beckerle et al., 1987). We cleaved chicken gizzard talin with chymotrypsin and separated the two fragments by high pressure liquid chromatography. Subsequent galactosylation with UDP-[3H]galactose and galac-

---

2 J. Hagmann, M. Grob, and M. M. Burger, unpublished observations.
toxyltransferase demonstrated that the 190-kDa fragment, but not the 47-kDa fragment, carry terminal GlcNAc moieties. We sequenced the first 16 amino acids of the 47-kDa fragment and obtained the sequence XALSLKIXIXNVVKTM. With the exception of 3 residues which could not be identified (X), the remaining 13 amino acids match the amino-terminal sequence of murine talin, beginning with the 2nd residue, published by Rees et al. (1990). This confirms that the 47-kDa fragment is the amino-terminal head of the molecule and that the glycosylation sites of talin must reside in the large 190-kDa tail.

We then digested the labeled 190-kDa fragment with trypsin and V8 protease and separated the fragments as described under “Experimental Procedures.” About 120 peaks were identified by measuring the optical density at 215 nm. Three peaks were found to be labeled (Fig. 3). The weakly labeled peak (peak 1 in Fig. 3) turned out to consist of the unlabeled fragment NIFSSMGDAGE and a very small radioactive fraction which could not be sequenced. The sequenced fragment fully corresponds to amino acids 811-821 of mouse talin (Rees et al., 1990). The contamination of peak 1 was obtained repeatedly. Peaks 2 and 3 were pure and could be sequenced. The sequences were compared with the amino acid sequence of murine talin published by Rees et al. (1990). Fig. 4 shows that the glycosylated sites are situated in the carboxyl-terminal half of the tail domain of talin. In both cases, the putative glycosylation sites are threonine residues, surrounded by PmQ and QLTND, respectively. Moreover, both peptides contain the sequence ANQ. The positions occupied by amino acid residues we could not identify (X) correspond to cysteine residues in the mouse talin sequence. Since our method of sequencing doesn’t detect cysteine residues, this amino acid most likely occupies the positions of X in chicken gizzard talin as well.

Glycosylation of Mammalian Talin—No labeling could be detected in talin isolated from human platelets and subjected to galactosylation in the presence of UDP-[3H]galactose, even after prolonged exposure of the film (Fig. 5). Following activation of platelets, talin is translocated from the cytosol to the plasma membrane (Beckerle et al., 1989). In order to find out whether glycosylation of talin is correlated with membrane localization, we performed the galactosylation reaction with talin isolated from thrombin stimulated platelets. Fig. 6 shows that activation did not lead to glycosylation of talin. This could be due to a difference between mammals and birds or to tissue specificity. In order to investigate this question, we isolated talin from mammalian (porcine) smooth muscle and subjected it to galactosylation. Contrary to platelet talin, porcine smooth muscle talin is glycosylated (Fig. 5). We calculated that 0.03 mol of [3H]galactose was incorporated into 1 mol of talin, indicating that 3% or less of total porcine stomach talin carries terminal GlcNAc residues. The results described in this paragraph support the idea that O-glycosylation of talin is tissue-specific.

DISCUSSION

When Torres and Hart (1984) labeled the proteins of lymphocytes with UDP-[3H]galactose and galactosyltransferase,
they discovered that the majority of the label was attached to intracellular proteins. The observation was extended to other tissues, and it was found that the nucleus and the cytosol were especially enriched in glycosylated proteins (Holt and Hart, 1986). In all cases examined so far, it turned out that the galactose residue is attached to a single GlcNAc molecule O-linked to either serine or, less often, threonine (reviewed in Hart et al., 1988, 1989a, 1989b). Subsequently, several intracellular glycosylated proteins were identified and further characterized. It was found that transcription factors (Jackson and Tjian, 1988) and nuclear pore proteins (Holt et al., 1987b) carry clusters of O-linked GlcNAc moieties which are highly immunogenic and bind wheat germ agglutinin. In addition, there is evidence that glycosylation of these proteins plays a role in transcription and in nuclear transport (Jackson and Tjian, 1988; Finlay et al., 1987).

The study of cytosolic glycosylated proteins is less advanced. One of the few glycosylated proteins to have been identified is protein 4.1 (Holt et al., 1987a), which links actin filaments to the integral plasma membrane protein glycophorin. Unlike transcription factors and nuclear pore proteins, protein 4.1 is only weakly glycosylated by galactosyltransferase (0.21 mol of galactose added per mol of protein), indicating that at most 21% of protein 4.1 contains accessible O-GlcNAc moieties.

Like protein 4.1, talin is implicated in binding actin filaments to the plasma membrane (reviewed in Beckerle and Yeh, 1990). Here we show that talin is also labeled by UDP-[3H]galactose and galactosyltransferase. β-Elimination and subsequent analysis of the carbohydrate moiety indicates that, as in the cases examined so far, galactose is attached to O-linked GlcNAc residues residing on serine or threonine. Similar to what has been reported for protein 4.1, only a fraction of the chicken gizzard talin molecules are labeled by the procedure: 6% or less, depending on how many accessible GlcNAc residues are incorporated into a single molecule of talin. We don’t know whether the remaining 94% (or more) of talin molecules are not glycosylated, or whether GlcNAc is not accessible to the galactosyltransferase, because it is hidden inside the molecule or blocked by an unknown group. When we attempted to bind chicken talin to a Sepharose column coupled with wheat germ agglutinin, it was not retained, and the talin in the flow-through could be labeled by galactosyltransferase to the same extent as before, indicating that no minor fraction of heavily galactosylated protein was irreversibly retained (data not shown). The lack of binding to wheat germ agglutinin indicates that, unlike on nuclear pore proteins and on transcription factors, sugar residues on talin are not clustered. In order to explore this question further, we analyzed short peptide sequences of talin which were labeled with [3H]galactose.

The amino acid sequence of mouse talin has recently been published (Rees et al., 1990). Three domains can be distinguished: a polar amino-terminal head comprising the first 600 residues, a long tail enriched in alanine, and a highly charged group of 60 carboxyl-terminal amino acids. The head domains of talin and of protein 4.1 are homologous, and they share the polarity of the carboxyl terminus. However, they differ in their tail domains which, in the case of protein 4.1, is much shorter. We first established that the glycosylation sites of talin reside in the carboxyl-terminal 190-kDa tail fragment generated by digestion with chymotrypsin. During subsequent analysis of short peptides generated from the 190-kDa fragment, we could identify two glycosylated sequences which closely matched corresponding sequences in mouse talin. The distribution of the labeled peptides on the 190-kDa fragment, and the fact that only 1 threonine per peptide was identified as a potential glycosylation site, excludes clustering of GlcNAc residues. The potential GlcNAc accepting threonine residues are embedded in the sequences PAXTG and NFLTNTDY. As explained under “Results,” the unknown residue X most likely is cysteine. The sequences therefore differ from the arrangement which is common to O-GlcNAc sites known so far (Hart et al., 1989b), where the acceptor residue lies next to an acidic amino acid and within at most 3 residues from a proline. We don’t know whether the fact that both peptides contain the only two ANQ sequences found in talin is significant. On protein 4.1, a GlcNAc-accepting region of 20 amino acids was identified on the carboxyl-terminal half of the tail region (Inaba and Maede, 1989). Our observation therefore adds to the similarities between talin and protein 4.1; besides the homologies between the head domains and the polarity of the carboxyl terminus, their tail domains are glycosylated. Talin differs from protein 4.1, however, in that the latter possesses 12 potential GlcNAc-accepting serine or threonine residues within the glycosylated 20-amino acid region.

The function of O-GlcNAc residues on cytosolic proteins is still obscure. The tail domains of both talin and protein 4.1 interact with other cytoskeletal proteins, with vinculin in the case of talin (Nuckolls et al., 1990), and with spectrin/actin in the case of protein 4.1. O-GlcNAc residues could contribute to these interactions. Alternatively, two other modulatory functions which have been suggested for glycosylation could apply: (i) talin can be phosphorylated, and glycosylation of threonine might prevent phosphorylation of these residues. This possibility is less likely, since, contrary to what has been found in other proteins, the glycosylated residues of chicken gizzard talin are not surrounded by amino acids typical of phosphorylation sites; (ii) talin is a substrate of the Ca2+-dependent protease calpain. Glycosylation might modulate the susceptibility of talin to proteases. However, in vitro experiments performed by us gave no evidence for this hypothesis. A third possibility is suggested by our finding that galactosylation of talin is tissue-specific; it is found in chicken gizzard smooth muscle and porcine smooth muscle, but not in human platelet talin. It has been shown that talin is located in the cytosol of resting platelets and becomes membrane-attached after stimulation (Beckerle et al., 1989). We therefore examined the possibility that glycosylation of talin is the result of close proximity to the membrane bound UDP-GlcNAc:polypeptide O-GlcNAc transferase. However, activation with thrombin which leads to platelet aggregation and translation of talin didn’t produce talin that could be labeled by galactosylation (Fig. 6). Interestingly, the behavior of platelet talin differs from talin found in other cell types in at least one respect: in all tissue culture cells we and others

3 J. Hagmann, M. Grob, and M. M. Burger, unpublished data.
examine, talin is found together with vinculin in focal contacts. In platelets, on the other hand, talin is absent from the vinculin-rich patches at the ends of stress fibers. Instead, it is distributed evenly on the plasma membrane and concentrated at the peripheral margin of platelets spread on a solid surface (Nachmias and Golla, 1991). Whether this difference in localization is related to the differing glycosylation status is presently under investigation.

Acknowledgments—We thank Dr. J. Hofsteenge and R. Matthies for expert advice and help with purifying and sequencing the peptide fragments.

REFERENCES

Beckerle, M. C., and Yeh, R. K. (1990) Cell Motil. Cytoskeleton 16, 7-13
Beckerle, M. C., Burridge, K., DeMartino, G. N., and Croall, D. E. (1987) Cell 51, 569-577
Beckerle, M. C., Miller, D. E., Bertagnolli, M. E., and Locke, S. J. (1989) J. Cell Biol. 109, 3383-3346
Belkin, A. M., Zhidkova, N. I., and Koteliansky, V. E. (1986) FEBS Lett. 200, 32-36
Burridge, K., and Connell, K. L. (1983) J. Cell Biol. 97, 359-367
Finlay, D. R., Newmeyer, D. D., Price, T. M., and Forbes, D. J. (1987) J. Cell Biol. 104, 189-200
Feramisco, J. R., and Burridge, K. (1980) J. Biol. Chem. 265, 1194-1199
Hart, G. W., Holt, G. D., and Haltiwanger, R. S. (1988a) Annu. Rev. Biochem. 58, 541-574
Hart, G. W., Haltiwanger, R. S., Holt, G. D., and Kelly, W. G. (1989b) Ciba Found. Symp. 145, 102-118
Holt, G. D., and Hart, G. W. (1988) J. Biol. Chem. 263, 8049-8057
Holt, G. D., Haltiwanger, R. S., Torres, C.-R., and Hart, G. W. (1987a) J. Biol. Chem. 262, 14847-14850
Holt, G. D., Snow, C., Senior, A., Haltiwanger, R. S., Gerace, L., and Hart, G. W. (1987b) J. Cell Biol. 104, 1157-1164
Horwitz, A., Dugnan, K., Buck, C., Beckerle, M. C., and Burridge, K. (1986) Nature 320, 531-533
Inaba, M., and Maede, Y. (1989) J. Biol. Chem. 264, 18149-18155
Jackson, S. P., and Tjian, R. (1988) Cell 55, 129-133
Kupfer, A., and Singer, S. J. (1989) J. Exp. Med. 170, 1697-1713
Laemmli, U. K. (1970) Nature 227, 680-685
Meyer, D. I., and Burger, M. M. (1976) Biochim. Biophys. Acta 443, 428-436
Molony, L., McCaslin, D., Abernethy, J., Paschal, B., and Burridge, K. (1987) J. Biol. Chem. 262, 7790-7798
Nachmias, V. T., and Golla, R. (1991) Cell Motil. Cytoskeleton 20, 190-202
Nuckolls, G. H., Turner, C. E., and Burridge, K. (1990) J. Cell Biol. 110, 1635-1644
Jackson, S. P., and Tjian, R. (1988) Cell 56, 125-133
O'Halloran, T., Molony, L., and Burridge, K. (1986) Methods Enzymol. 134, 69-77
Rees, D. J. G., Ades, S. E., Singer, S. J., and Hynes, R. O. (1990) Nature 347, 586-589
Rochlin, M. W., Chen, Q., Tobler, M., Turner, C. E., Burridge, K., and Peng, H. B. (1989) J. Cell Sci. 92, 461-472
Sealock, R., Paschal, B., Beckerle, M., and Burridge, K. (1986) Exp. Cell Res. 163, 143-169
Torres, C.-R., and Hart, G. W. (1984) J. Biol. Chem. 259, 3308-3317
Turner, C. E., Pevault, F. M., and Burridge, K. (1989) J. Biol. Chem. 264, 11938-11944
Wilkins, J. A., and Lin, S. (1986) J. Cell Biol. 102, 1085-1092