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Effects of ligands on the stability of tissue transglutaminase: studies in vitro suggest possible modulation by ligands of protein turn-over in vivo*

Review Article

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Summary. Tissue transglutaminase catalyzes irreversible post-translational modification of specific protein substrates by either crosslinkage or incorporation of primary amines into glutamine residues, through glutamyl-amide isopeptide bonds. Modulation in vivo of these reactions (collectively called ''transamidation'') is brought about by both ligand dependent effects (chiefly, activation by calcium and inhibition by GTP) as well as by variation in enzyme tissue levels by transcriptional effects. Accumulating observations that the enzyme stability in vitro is greatly affected by interaction with ligands led us to postulate that also the turn-over in vivo might be modulated by ligands opening new scenarios on the regulation of the tissue transamidating activity. This proposal is consistent with data obtained in in vitro cell culture systems and has important implications for the expression of activity in vivo.

Keywords: Transglutaminase – Protein cross-links – GTP – Polyamines

Reactions catalyzed by transglutaminases and their physiologic roles

Transglutaminases catalyze protein posttranslational modification at peptidyl glutaminyl-amido groups through acyl transfer to an acceptor primary amine thus converting a primary into a secondary amide function via a glutamyl-thioester intermediate as reviewed recently (Griffin et al., 2002; Beninati and Piacentini, 2004). The amine donor can be represented by either the ε-aminogroup of a peptidyl lysine residue or alternatively a soluble amine (usually a polyamine or histamine) (Folk et al., 1980). In both instances isopeptide bonds are formed resulting into the production of either inter/intramolecular crosslinked proteins or of proteins postranslationally modified at glutamine residues. The alternative release of these products arises from a strict specificity for recognition of peptidyl glutamine residues (but rules governing selection of only a tiny fraction of surface exposed glutamines are still unclear) (Esposito and Caputo, 2005) and a rather loose specificity for the amine acyl-acceptor. Depending on experimental conditions, e.g. absence of alternative amine acceptors, water can also act as acyl-accepting nucleophile with hydrolysis of glutamine to glutamate residues, but this is a rather rare event, although it might be physiopathologically relevant in the onset of coeliac disease (Koning et al., 2005).

In most instances, the products of the transglutaminase reaction are insoluble protein aggregates which can be cleared only through massive protein breakdown, despite the reversibility of the reaction has been demonstrated in vitro employing moderately modified peptide models (Parameswaran et al., 1997). The stability of the glutamyl-isopeptide linkage is well appreciated and actually this bond was selected in nature to mark irreversible modification of proteins entering degradative pathways, as it happens in the ubiquitin-related proteolysis (Dohmen, 2004). Only one enzyme has been presently identified capable to degrade this linkage, γ-glutamyl cyclotransferase; it is present in considerable amounts in mammalian kidney and is active only on isolated isodipeptide released after

* The author dedicates this contribution to the memory of Mrs Laura Pasquali Bergamini, who died October 27, 2005.

extensive proteolysis of isodipeptide-crosslinked proteins (Fink et al., 1980).

The effective irreversibility of transamidation strongly requires that enzymes involved in this reaction are carefully regulated. In mammals 9 transglutaminase isoforms have been described at the genomic level (Grenard et al., 2001) and some of them (the tissular forms 1–5, and the plasma clotting Factor XIII) have been isolated and investigated also at the protein level. These studies revealed that activity is tightly regulated via different mechanisms for the distinct isoforms, although all mammalian transglutaminases are strictly dependent on calcium ions which interacts directly with the enzyme, without involvement of any calcium-binding protein, such as calmodulin. In addition each isoenzyme displays specific regulatory features (see also Griffin et al., 2002; Beninati and Piacentini, 2004), which include proteolysis by thrombin and calcium dependent dissociation of an inhibitory subunit in the case of plasma transglutaminase Factor XIII, involved in blood clotting and wound healing; activation by proteolysis and association to the plasma membrane, limited proteolysis and additionally regulation by GTP, in the case of type-1, type-3 and type-5 transglutaminase respectively (all these enzymes are present in the skin and are involved in terminal differentiation of keratinocytes); type-4 transglutaminase (present in prostatic secretions particularly in rodents) is largely independent of added calcium ions because it contains firmly-bound calcium, so that activity in vivo is essentially dependent only on availability of substrate proteins secreted into the prostatic fluid by the seminal vesicles (Esposito and Caputo, 2005).

I held for the last the isoform type-2 transglutaminase, on which I will focus this discussion, because its regulation and its physiologic function are still matter of discussion. At difference with the other isoenzymes, this protein is clearly more complicate because it harbours at distinct sites activities for transamidation, GTP hydrolysis (i.e. GTP-ase activity, acting as a monomeric G-protein for signal transduction related to phosphoinositides hydrolysis), protein-disulfide isomerization and possibly even serine protein kinase activity, as recently suggested (Mishra and Murphy, 2006). Type-2 transglutaminase also harbours binding sites for anchorage at the external surface of the plasma membrane, acting as an exoenzyme, along with its ability to modify intracellular proteins. From a physiologic point of view it has been proposed that this isoenzyme is involved in an array of processes as described in the next session (see Griffin et al., 2002; Bergamini et al., 2005, for recent discussions).

Regulation of type-2 transglutaminase activity by ligands

The main transglutaminase isoenzyme is type-2 (or simply ''tissue'') transglutaminase, which is widespread and expressed at particularly high levels in endothelial cells which account for most of the enzyme present in resting organs. In relation to subcellular distribution, it is important to note that while the vast majority of the protein is present in the cytosolic compartment, a moderate but significant fraction is associated with plasma membranes, where it can translocate to the external surface by unknown mechanisms, becoming an active exoenzyme.

As stated in the previous section, type-2 transglutaminase is a multifunctional enzyme, with four distinct activities: Transamidase, GTP-ase, protein kinse and Protein Disulfide Isomerase (PDI) activity (Chandrashekar et al., 1998). Properties of the protein kinase and of the PDI activity are still largely unexplored, particularly in terms of regulation, and this issue will not be dealt further in the present occasion. In contrast much is known on the transamidating and the hydrolytic activity. The pattern emerging from the available studies indicates that the transamidating activity of cytosolic transglutaminase depends in vitro on the exposition to extremely high concentrations of calcium (about 1 millimolar) and that GTP is an effective inhibitor of enzyme activity, at low calcium concentration. Conversely, calcium apparently inhibits hydrolysis of GTP leading researchers to the conclusion that these activities must be switched on/off in alternative way, largely in relation to the availability of calcium. Advanced analysis of the molecular basis of these regulatory effects (see Griffin et al., 2002, for a detailed discussion) revealed that the enzyme active site for transamidation is located deeply within domain 2 and that it is not available to the glutamyl-substrate protein because of steric hindrance brought about through overlayering by the tightly interacting domains 3 and 4. In contrast the GTP-ase site is freely accessible to the substrate at the enzyme surface. This structural organization is altered by interaction with calcium which promotes movement of domains 3 and 4 far away from the active site, allowing catalysis to take place at the transamidation site. Under these conditions the reactivity of active site Cysteine 277 (which is constantly kept at a basal reactivity state through either hydrogen bonding to Tyr 516 or through formation of a disulphide bridge with a thiol group) increases several folds, rendering the enzyme fully active. Thus type-2 transglutaminase is inactive in protein crosslinking (i.e. it is a ''cryptic'' enzyme) unless high concentrations of calcium

are available to overwhelm the domain–domain interactions which take place in the ''resting'' protein, through a conformational change. This pattern is complicated by the actions of additional modulators (NO-donor agents and sphingosylphosphoryl choline) which tune the effects of calcium, but these processes were investigated only from the kinetic, not from the structural point of view so that their relevance cannot yet be fully appreciated.

Another point of extreme relevance is represented by the subcellular distribution of type-2 transglutaminase. This enzyme is largely present in the cytosolic compartment, but a consistent fraction is associated with the plasma membrane (some of this is actually exposed at the external surface, following translocation by unknown mechanisms). Reports are also appearing supporting the presence of tiny amounts in the mitochondrion (particularly in the nervous tissue) and in the nucleus. Membrane associated transglutaminase has probably distinct functional roles from the bulk cytosolic protein: for instance the externalized enzyme is apparently constitutively active in the transamidating reaction at the high concentrations of calcium and the low concentrations of nucleotide triphosphates which characterize the extracellular compartment. Under these conditions, tissue transglutaminase interacts easily with fibronectin, integrins and heparan-sulphates driving polymerization and assembly of a number of extracellular matrix proteins, including fibrinogen and fibronectin, controlling in addition processing and secretion of cytokines such as TGFβ1. Intracellular membrane-associated transglutaminase has further different functions, serving as a large monomeric G-protein (usually indicated as G_h), to transduce signals of adrenergic agents, within the frame of cell signalling network. In this perspective it is important to remind the reader that the concerted regulation of tissue transglutaminase by GTP/GDP and by calcium ions, which are considered the main modulators of activity, force the enzyme towards protein crosslinkage (under the action of calcium) or towards GTPase and signalling activity (in the presence of GTP), in a mutually exclusive way. In relation to regulation by nitrosylating agents and by sphingosylphosphorylcholine, it must be underlined that regulation by nitrosylation is probably extremely important in the endothelium, which expresses the enzyme at high levels. Another point of active research in relation to features of extracellular membrane-bound transglutaminase is represented by the sensitivity to metallo proteinases (in particular forms 1 and 2 of the MMMP family) (Belkin et al., 2004), which cleave and inactivate the enzyme (also see below).

In relation to the functional aspects, the enzyme is expected to be involved in different processes depending on the activity which is actually switched-on. As detailed above the transamidating activity is apparently involved in both intracellular and extracellular protein crosslinkage (and eventually in conjugation of proteins with polyamines). The role of transamidation is dependent on the cell compartment where the reaction takes place and obviously on the alteration of properties the substrate protein undergoes upon modification. Thus in the case of intracellular transglutaminase it is postulated that crosslinkage of intracellular proteins is activated only in cells suffering irreversible damage by whatever challenge within the programme of cell death by the apoptotic pathway (reviewed by Griffin et al., 2002), although it cannot be completely excluded that additional factors contribute to regulation of activity by ''physiological'' concentrations of ligands, at least in situ in permeabilized cells (Smethurst and Griffin, 1996). Much interest in this perspective is focusing on the small fraction of activity present inside the mitochondrion, at the light of the role played by the organelle in this process. In contrast, transamidation in the extracellular compartment is related to the maturation of proteins of the extracellular matrix, to which transglutaminase is anchored because of its affinity for fibronectin and for integrins. Under these conditions, the transamidating activity is presumably constitutively ''on'' because of the concentrations of regulatory ligands to which the enzyme is constantly exposed. In this perspective it is likely that proteolysis is a major determinant of activity at the external cell surface (Belkin et al., 2004; Bergamini et al., 2005).

Still different functions are ascribed to intracellular transglutaminase during normal cell life, under conditions in which the transamidating activity is switched-off, while the GTP-ase activity is involved in transduction of extracellular signals, particularly those involving the phospholipase-C, phosphoinositides and possibly the Akt-protein kinase pathway. As already pointed out, under these conditions, type-2 transglutaminase is believed to act as a monomeric G-protein, to associate with an additional membrane protein, Calreticulin (Feng et al., 1999), and to transduce α-adrenergic and thromboxane mediated signals. During this alternative metabolic activity, transglutaminase G_h is characterized by high GTP-ase and minimal transamidating activity and is involved in transmission of cell survival signals (Mian et al., 1995) and in tissue growth, such as it happens in genetically transformed mice overexpressing transglutaminase in the cardiac muscle (Small et al., 1999). Even if the proliferative effects are

mainly ascribed to the GTP-ase activity, this issue is still open (see also the following section). The alternative approach to investigate the proliferative actions of tissue transglutaminase producing specific KO mice did not provide significant information because the animals do not display significant phenotypic defects, possibly because of an internal compensation by other enzyme isoforms, although a general decline in phagocytosis activity have been documented.

Transcriptional regulation of type-2 transglutaminase tissue levels

Another point worth underlining in relation to the present discussion is related to the expression levels of the TG2 protein within tissues. In ''normal'' resting tissues expression of the protein is relatively low and the protein detectable by immunohistochemical techniques is largely present in vascular structures, as proved elegantly in extensive investigations (Thomazy and Fesus, 1989). However tissue concentrations of type-2 transglutaminase can be largely affected by treatment with inducers or by the onset of certain pathologic states, e.g. apoptosis, tissue fibrosis and atherosclerosis. Inducers include retinoids, particularly *all*-trans retinoic acid, through transcription of the transglutaminase structural gene and accumulation of mRNA in tissues (Nagy et al., 1997).

Also inflammatory cytokines (IL-6, TGFα and TGFβ, Interferon γ) appear to be active inducers, suggesting that the reported increase in transglutaminase activity in inflammatory states might be related to these effects. Among steroid hormones, some appear to enhance expression of tissue transglutaminase (notably vitamin D and progesterone) while others depress it (particularly glucocorticoids in the skin). As mentioned above, hyperexpression of tissue transglutaminase can also promote growth effects in experimental animals, as demonstrated by the cardiac hypertrophy occurring in transgenic mice overexpressing the enzyme (37-fold increase over control animals), probably through the transamidating, not the G-protein effects as originally expected, and conversely that pressure overload (through aortic constriction) can likewise lead to enzyme overexpression during heart failure. In other words, pressure overload can lead to increased enzyme and, conversely, increased enzyme expression promotes heart hypertrophy, stressing the mutual relationship between working pressure and enzyme expression, which is of great importance for cardiac activity.

Another stimulus possibly leading to controlled (stimulated) expression levels of TG2, of increased potential interest in cardiovascular diseases, is represented by ROS. For instance lymphocytes and fibroblasts exposed to increased levels of reactive oxygen species (generated either enzymatically by xanthine oxidase or by addition of H_2O_2 to the culture medium) undergo intensive growth effects along with increased transglutaminase activity.

Major effects of stimulated expression of type-2 transglutaminase are classically observed in cells undergoing apoptosis, as suggested by Fesüs and associates (see Fesüs and Szondi, 2005, for a recent review). In the original experiments, authors investigated the role of type-2 transglutaminase in regression of liver hypertrophy induced by administration of lead salts. Results demonstrated that cellular levels of transglutaminase increased several folds after stopping the experimental treatment, with a high wave of formation of isopeptide crosslinked proteins in the tissue and an increased rate of cell death. These findings were indicative of accelerated synthesis and increased activity in situ of the enzyme, which modified extensively cytoskeletal proteins, leading to the formation of apoptotic bodies (insoluble to detergents), along with the usual internucleosomal cleavage of nuclear DNA. These findings were reproduced also in other experimental models, suggesting that the increased transamidating activity usually observed in cells undergoing apoptosis is the result of both enzyme induction and enzyme activation by variation in tissue concentrations of ligands.

Proteolysis and regulation of type-2 transglutaminase tissue levels

The main aim of this discussion is to shed light on the possibility that degradation of transglutaminases by proteinases might contribute to regulate enzyme levels in tissues and thereby tissular transamidating activity itself. The general issue of proteolysis and regulation of specific proteins levels has been extensively investigated since the early '70s, starting through the efforts of R.T. Schimke (see his classic review, dated 1973) who pointed out the existence of short- and long-living proteins in cells. The tissue half-life of proteins can range from minutes up to weeks, largely depending on development and differentiation of the tissue under investigation as well as on external challenges, e.g. nutritional and endocrine stimula. Obviously, the contribution of regulated proteolysis in modulating levels of specific proteins in tissues is quantitatively more relevant for short-living proteins and transglutaminases do not certainly escape this general law.

A crucial parameter in this perspective is represented by the half-life of proteins in vivo, which can eventually be affected by interaction with ligands and with regulatory proteins (Li and Coffino, 1994). In the case of transglutaminases, data in this perspective are extremely fragmentary and not truly focused to solve this question. Furthermore experimental results are available only in the case of tissue transglutaminase which is believed to belong to short-living proteins, with an average half-life of only 11 hours (M. Griffin, personal observation, but see quotation in Griffin et al., 2002). On line with these findings, it must be reminded that several reports have appeared in the literature to document the appearance of active and inactive forms of transglutaminase in tissular extracts. For instance as early as 1975, Chung described the chromatographic resolution of multiple enzyme forms of different peptide size in extracts of human and guinea pig tissues. All these isoforms were detected by activity assay, so that they were at least partially active, although possibly undergoing proteolysis at the time of isolation. Interpretation of these results however is not straight-forward since it is known that ''small'' forms of transglutaminase (with simplified regulation, since they are usually insentive to inhibition by GTP) may arise also through translation of mRNA generated by ''exon shuffling'' of the genomic DNA (Festoff et al., 2002). The coexistence of active and inactive forms within tissues is in any case well known, particularly within neoplastic tissues, but their relationship to proteolysis has not been investigated. In some instances this was proved clearly as in the nervous tissue (Zhang et al., 1998a) and in lymphocytes undergoing apoptosis (Fabbi et al., 1999), where proteolyzed forms can arise by intracellular degradation by μ-calpain and by caspase 3, respectively. As it will be detailed in the next section, it is likely that regulation of cleavage and of turn-over of intracellular transglutaminase is achieved through interaction with the physiologically relevant modulators Ca^{2+} and GTP, as proved in vitro in cultured cells, pharmacologically manipulated to modify the concentration of these ligands (Zhang et al., 1998b).

As already pointed, proteolysis is particularly relevant in the case of extracellular membrane-bound transglutaminase, since this is the only mechanism which is supposed to regulate effectively the transamidating activity of the enzyme during assembly of the proteins of the extracellular matrix through the activity of Membrane Metallo-Proteinases and of their specific inhibitors. These processes are believed to be important physiologically in the regulation of chronic inflammation and eventually of tissue fibrosis.

Ligands and stability of type-2 transglutaminase in vitro

As mentioned above, there is evidence that calcium and GTP modulate turnover of tissue transglutaminase affecting proteolysis of the enzyme in situ in cultured cells. The most convincing results were obtained by Zhang et al. (1998b). These authors observed that treatment of neuroblastoma cells with maitotoxin and tiazofurin (respectively to increase calcium and to decrease GTP concentration through effects on membrane permeability and inhibition of GTP biosynthesis) modified by one side catalytic activity, as expected from results obtained in vitro, by the other altered the stability of the protein in a calpain dependent way. Increased calcium levels promoted enzyme proteolysis while GTP protected the protein from degradation.

In a general perspective, analogous results about opposite effects of calcium and GTP on the stability of type-2 transglutaminase were obtained in vitro with the purified protein. This is particularly interesting because it indicates that proteins to which transglutaminase is reported to display high affinity within cells, e.g. fibronectin and calreticulin (possibly in relation to membrane localization) (Feng et al., 1998) or cytoskeletal proteins and cytosolic enzymes, e.g. glutatione-reductase (Piredda et al., 1999), do not greatly affect the protein stability in vivo, although they are reported to affect activity (see Feng et al., 1998).

In the investigations in vitro, it was reported that calcium stimulated and GTP protected against inactivation during treatment with several commercial proteinases (Bergamini, 1988; Casadio et al., 1999), or exposure to different denaturating conditions as thermal treatment (as demonstrated for instance by Differential Scanning Calorimetry, see Bergamini et al., submitted) or incubation with guanidine (Di Venere et al., 2000; Bergamini and Spisni, in preparation), or even during incubation with chemicals reactive against aminoacids involved in the catalytic mechanism (unpublished observations). Since the enzyme responds similarly to all these different challenges, it is tempting to imagine that this behaviour stems from an alteration in the overall protein stability, triggered by binding of the ligands, not from specific denaturant-dependent effects. Recent advances in understanding the molecular physiology of type-2 transglutaminase enlighten these phenomena (Casadio et al., 1999; Mariani et al., 2000; Griffin et al., 2002). As outlined above, molecular mechanisms of regulation of type-2 transglutaminase activity involve movements in domains 2 and 3 across an exposed loop, which behaves like a hinge. During the

conformational change domains 3 and 4 shift far away from the central region of protein, weakening the reciprocal interactions between the N- and the C-terminal domain pairs, in the case of interaction with calcium, and conversely reinforcing them in the presence of GTP. The alterations in the reciprocal interaction between N- and C-terminal domains are consistent with the occurrence of more or less stable conformers, explaining the behaviour towards chemical and physical denaturants. Interpretation of the effects of ligands on proteolysis, which is obviously the normal mechanism of turn-over of transglutaminase in tissues, is probably quite different, because it is known that changes in local flexibility at the exposed peptide chains (which are the targets of proteinases) are the main regulator of proteolysis in native proteins (Fontana, 1991). Consequently, the effects of calcium and of GTP as modulators of transglutaminase breakdown must be ascribed to changes in the flexibility of the peptide chain backbone rather than to modifications domain–domain interaction forces which are involved in the other forms of physical and chemical protein denaturations we have taken into account. Other tissular components, e.g. chaperonins or osmolytes (Bolen and Baskakov, 2001) might eventually play a role in these phenomena, but these parameters have never been investigated until now.

Conclusions

In the above discussion I tried to summarize recent evidences on the potential roles of proteinases as effectors of type-2 transglutaminase turnover in mammalian cells, in a process regulated by the ligands calcium and GTP. Someway similar processes of ligand-induced regulation of tissue levels occur also in the case of other transglutaminases, such as type-1 transglutaminase, whose expression is stimulated by calcium ions, and type-3 transglutaminase, which is modulated by proteolysis in the presence of calcium, resulting into irreversible enzyme activation (instead of inactivation as for type-2 transglutaminase) but this is again an example of irreversible processing towards degradation.

Taking into account the cumulated evidences of commitment of type-2 transglutaminase in apoptosis and the observations that activity is observed also at relatively physiological levels of the ligands (compare the results by Smethurst and Griffin, 1996, already cited) and the observation that the membrane phospholipid sphingosylphosphocholine sensitizes type-2 transglutaminase towards calcium activation, it is clear that activation of the enzyme is not always associated with the onset of apoptosis. In this perspective, the facilitated enzyme degradation through proteolysis is a mechanism of protection against inappropriate enzyme activation, under submaximal stimulation by calcium. It is thus clear that proteinases have potentials for controlling enzyme tissue levels, effective on both intracellular and extracellular forms. Careful measurements of enzyme life span are required to substantiate this working hypothesis.

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