Aldehyde fixation of tissues often adversely affects the reactivity of cellular proteins with antibodies. A most commonly used retrieval technique in immunohistochemistry is high-temperature microwave heating of sections from formaldehyde-fixed and paraffin-embedded tissues. Here we report that pretreatment of paraffin and ultrathin cryosections with N-glycanase F to remove N-glycosidically linked oligosaccharides can result in a dramatic increase in specificity and intensity of immunogold labeling for sugar moieties present on O-glycosidically linked oligosaccharides. This is demonstrated in the immunolocalization of poly-α2,8 KDN (KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) of megalin in rat kidney. The mechanism of this retrieval procedure is most probably based on the elimination of sterical hindrance by large N-glycosidically linked oligosaccharides. Furthermore, we demonstrate that exposure of ultrathin cryosections to acidic conditions (pH 5.5) at ambient temperature prior to immunogold labeling can result in an increased labeling intensity. This effect was observed for megalin immunoreactive sites in proximal tubular epithelia of rat kidney. It is proposed that the mechanism of this retrieval procedure is based on the depolymerization of methylene and polymethylene bridges introduced by formaldehyde in the acidic milieu.

**Introduction**

Histochemical detection of proteins and specific sequences of oligosaccharide side chains with antibodies and lectins plays a key role in research and diagnostic pathology. Formaldehyde alone or in combination with glutaraldehyde represents the most commonly used chemical fixative and its adverse effects on the reactivity of cellular proteins with their respective specific antibodies have been amply documented (Brandtzaeg 1982; Griffiths 1993; Larsson 1988). Consequently, many protocols have been worked out to overcome the limitations imposed by aldehyde fixation, and additional effects probably caused by the “denaturation” occurring during dehydration in organic solvents and embedding in paraffin. Initial attempts to retrieve antigens in sections of formaldehyde-fixed and paraffin-embedded tissues involved protease digestion of the sections (Battifora and Koinski 1986; Huang et al. 1976) or immersion in water for extended periods of time (Puchtler and Meloan 1985). At present, the most commonly used retrieval technique in immunohistochemistry for diagnostic pathology is high-temperature microwave heating of sections from formaldehyde-fixed and paraffin-embedded tissues (Shi et al. 1997; Werner et al. 1996, for reviews). It is safe to say that the use of microwave heating has permitted a breakthrough in the application of immunohistochemistry in diagnostic pathology.

Immunocytochemistry as applied in research is an invaluable tool to elucidate the cellular and subcellular distribution of proteins and may aid studies aimed at understanding the protein’s function. As in diagnostic pathology, aldehyde fixation is commonly used and although the fixation conditions can be better controlled, antibodies may fail to recognize their respective antigens. Thus, polyclonal antibodies were raised against the formaldehyde-treated antigen to improve their reactivity toward formaldehyde-exposed cellular components (Harrach and Robeneck 1990). Brown and colleagues (1996) pointed out that some antibodies may produce excellent results when applied for immunoblotting, but no or only variable staining if used for immunocytochemistry. Immunoblotting involves protein separation by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) which is than followed by transfer of the proteins to membranes. Therefore, SDS-denatured proteins, in contrast to native proteins, seem to be preferentially recognized by the antibodies. To test this, Brown et al. (1996) pretreated cryostat sections or cell cultures which
had been fixed in parafomaldehyde-lysin periodate or parafomaldehyde with SDS. This resulted in a dramatic increase in intensity of immunofluorescence in many cases and in some other cases antigens only became detectable after SDS treatment. As might be expected, they also found antigens that were not affected by SDS pretreatment.

Here we show that pretreatment of paraffin sections or ultrathin cryosections from parafomaldehyde-glutaraldehyde-fixed tissue with N-glycanase F to remove N-glycosidically linked oligosaccharides of glycoproteins can result in a considerable improvement of both specificity and intensity of immunolabeling for oligosaccharidic structures present on O-glycosidically linked oligosaccharides. Furthermore, we report that exposure of ultrathin cryosections to low pH can augment immunogold labeling for certain membrane proteins.

Materials and methods

Purification of megalin and preparation of polyclonal antibodies

Megalin was isolated and purified from rat kidney microsomes by sequential DEAE-Sephaecel column chromatography, lectin lectin-Sepharose affinity chromatography and Sepharose S-400 column chromatography using an FPLC system (Ziaj et al. 1998). Fractions immunoreactive for poly α2,8 KDN (2-keto-3-deoxy-O-glycero-D-galacto-nononic acid) were pooled. In addition, megalin was purified by receptor associated protein (RAP)-affinity chromatography. For this purpose, a glutathione S-transferase (GST)-RAP/glutathione Sepharose 4B affinity column was prepared as described by Herz et al. (1991) with some modifications (Ziaj et al. 1998). In brief, for the amplification of RAP the 5′-ATCGAGGTCTGTTAGAACAGGGTCCTAC oligonucleotide was used as forward primer and the 5′-GAGCTCATTGTGCGAGCCTTTGGA-GAC oligonucleotide as reverse primer. Recombinant GST-RAP fusion protein expressed in E. coli was loaded on a glutathione-Sepharose 4B column. This column was used for the purification of megalin from rat kidney extracts.

For immunization, rabbits were injected with 100 µg of purified megalin in complete Freund’s adjuvant and received three booster injections (30 µg megalin each in incomplete Freund’s adjuvant) at 3-week intervals. Animals were bled after the third booster injection. An IgG fraction was prepared from the megalin antiserum using a protein A-Superose HR 10/2 column (Phar- macia, Uppsala, Sweden). By Western blotting, this anti-megalin IgG reacted with a single band at >350 kDa in kidney homogenates.

Tissue processing

Male adult Wistar rats (150–250 g body weight) were fasted overnight with free access to drinking water. They were anesthetized by an intraperitoneal injection of Nembutal (50 mg/kg body weight). The animals were perfused through the left cardiac ventricle with Hank’s balanced salt solution (pH 7.4) containing 3% weight). The animals were perfused through the left cardiac ventricle with Hank’s balanced salt solution (pH 7.4) containing 3% formaldehyde-fixed tissue with N-glycanase F to remove N-glycosidically linked oligosaccharides of glycoproteins can result in a considerable improvement of both specificity and intensity of immunolabeling for oligosaccharidic structures present on O-glycosidically linked oligosaccharides. Furthermore, we report that exposure of ultrathin cryosections to low pH can augment immunogold labeling for certain membrane proteins.

N-glycanase F treatment of tissue sections

Dewaxed and rehydrated paraffin sections were covered with acetate buffer (50 mM, pH 5.5), citrate buffer (50 mM, pH 6), or PBS (pH 7.4) containing 0.25 U/ml of recombinant N-glycanase F (Boehringer, Mannheim, Germany) and were incubated in a moist chamber at 37°C overnight. Afterwards, they were briefly washed with the corresponding incubation buffer and brought into PBS prior to immunolabeling (see below).

N-glycanase F treatment of tissue sections

Grids with the ultrathin cryosections were floated on droplets of acetate buffer (50 mM, pH 5.5), citrate buffer (50 mM, pH 6) or PBS (pH 7.4) containing 0.5 U/ml of recombinant N-glycanase F in a moist chamber for 4 h at 37°C. This was followed by transfer to droplets of the corresponding incubation buffer and conditioning on droplets of PBS containing 1% bovine serum albumin (BSA) and 0.01% Tween 20 for 10 min at ambient temperature. Controls included the incubation of paraffin or ultrathin cryosections in buffer without N-glycanase F.

Exposure of tissue sections to various pH values

Grids with the attached ultrathin cryosections were floated on droplets of the following buffers for 2 h at 37°C:

1. 50 mM phosphate buffer adjusted to pH 7.4, 5.8 and 5.5
2. 50 mM citrate-phosphate buffer adjusted to pH 7.4, 6.5, 6, 5.5, 5, 4.5, 4, and 3
3. 50 mM hydrochloric acid-potassium chloride buffer adjusted to pH 2 and 1.5.

This was followed by three brief rinses on droplets of PBS (pH 7.4). In addition, sets of grids with the ultrathin cryosections, treated as described above and rinsed with PBS, were floated on droplets of a freshly prepared aqueous borohydride solution (20 mM) for 10 min at ambient temperature and were subsequently washed by transferring them to droplets of PBS. Grids were subjected to immunolabeling as described below. In addition, paraffin sections were exposed to acetate buffer (50 mM, pH 5.5) for 2 h at ambient temperature prior to megalin immunolabeling.

Immunolabeling

Series of consecutive sections from various paraffin blocks, pretreated with N-glycanase F or not, or enzyme buffer alone, were conditioned with PBS (pH 7.4) containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20 for 20 min at ambient temperature. For the detection of poly α2,8 KDN immunoreactivity, they were then incubated with an IgM fraction (30 µg/ml in PBS, pH 7.4, containing 1% BSA, 0.05% Triton X-100, and 0.05% Tween 20) of the mAb kdm1a (Kusumi et al. 1994),