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Research paper

Alkaline phosphatase–immunoglobulin conjugate binds to lipids in vitro, independent of antibody selectivity

D. Lingwood *, J.S. Ballantyne

Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1

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Abstract

At present, alkaline phosphatase (AP) conjugates are major workhorses of immunological detection. However, APs are membrane bound enzymes, and therefore have the potential to interact with lipids. Using TLC overlay, we screened AP-conjugated immunoglobulins (IgGs), and AP-conjugated streptavidin, for their ability to bind sphingolipids and phospholipids non-specifically. Horseradish peroxidase (HRP)-conjugated IgG was tested as a negative control. AP-conjugates bound to all sphingolipids and phospholipids assayed, whereas no HRP-IgG binding was observed. AP conjugate-lipid binding could be reduced by pretreatment of chromatograms with polyisobutylmethacrylate. Addition of Tween 20 also abolished AP-lipid binding, except to lactosyl ceramide, suggesting a degree of specificity. This study serves to prevent spurious interpretation of AP-conjugate based binding assays, be they against purified lipids/lipid mixtures or tissue samples from which lipids have not been removed.
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Keywords: Alkaline phosphatase conjugate; Non-selective lipid binding

Mammalian alkaline phosphatase (AP) conjugates are widely used for detection in a number of immunological techniques including Western blotting, ELISA, immunohistochemistry and in situ hybridization. Use of AP-conjugates offers several distinct advantages including linear reaction rates (Fernley, 1971), insensitivity to antibacterial agents such as sodium azide (Van Noorden and Jonges, 1987) or thimerosal (Shavali et al., 1999), and control over some endogenous alkaline phosphatase activity by pretreatment with levamisole (Brenan and Bath, 1989). Although APs are broadly defined as phosphomonesterases with activity optima at alkaline pH, most are plasma membrane glycoproteins that therefore contain hydrophobic/lipid interacting moieties (Fernley, 1971). For example, human placental AP is a glycosylphosphatidylinositol anchored protein (Fukushima et al., 2003). It is therefore conceivable that the AP component of an AP-conjugate could interact with lipids. We tested this by overlaying three types of AP-conjugated IgGs and AP-conjugated streptavidin onto sphingolipids and phospholipids separated by TLC. As horseradish peroxidase (HRP) is not a membrane-derived protein, and is also a common enzyme conjugate, a parallel overlay with HRP-conjugated IgG was run as a negative control. We report that AP-conjugated systems bind lipids indiscriminately, and independently of their conjugate partner.

* Corresponding author. Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany.

E-mail address: lingwood@mpi-cbg.de (D. Lingwood).

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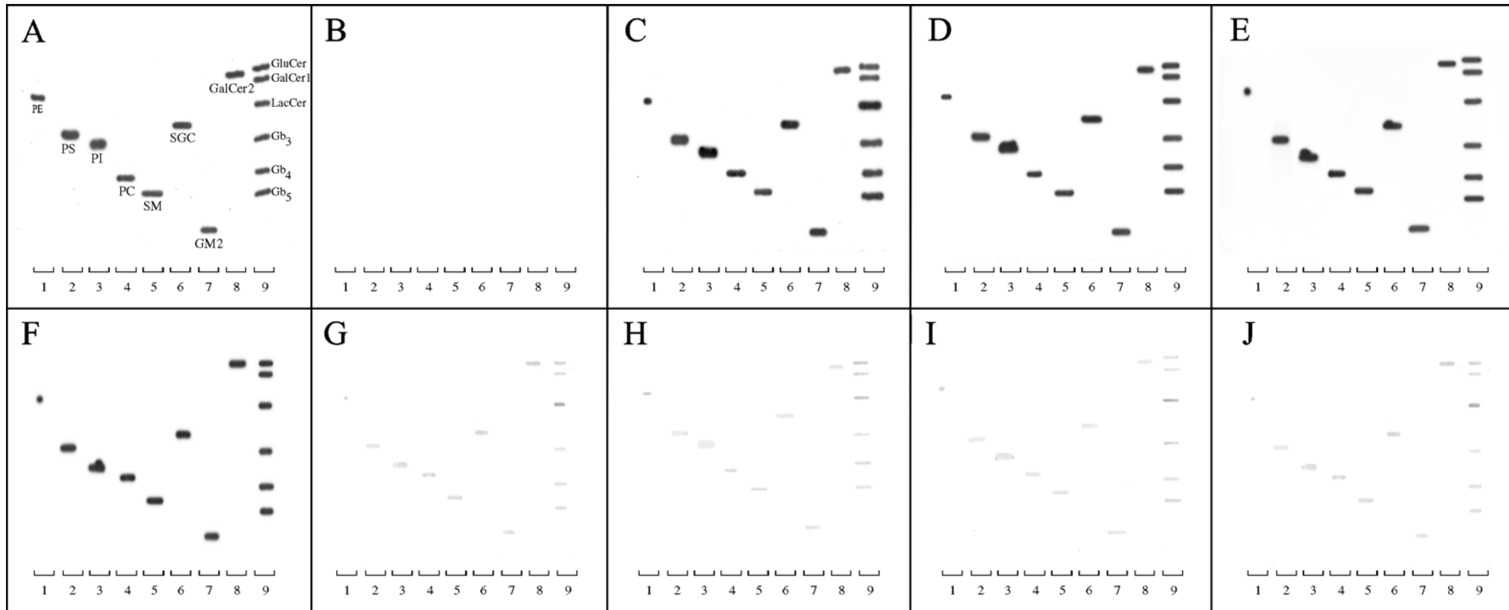

Fig. 1. Overlays of enzyme conjugates onto phospholipids and sphingolipids separated by TLC in a TBS system. Lanes 1–8 = PE, PS, PI, PC, SM, SGC, GM2, GalCer2, respectively; lane 9 = Gb_5 + Gb_4 + Gb_3 + LacCer + GalCer1 + GlcCer. A = reference plate (iodine exposed); B = HRP-GAM overlay; C = AP-GAM overlay; D = AP-AD overlay; E = AP-AF overlay; F = AP-SA overlay; G = AP-GAM overlay with PIBM; H = AP-AD overlay with PIBM; I = AP-AF overlay with PIBM; J = AP-SA overlay with PIBM.

Sphingolipid and phospholipid standards {phosphatidylethanolamine (PE); phosphatidylserine (PS); phosphatidylinositol (PI); phosphatidylcholine (PC); sphingomyelin (SM); sulfogalactosyl ceramide (SGC); GM2 ganglioside; type 2 galactosyl ceramide (GalCer2); globopentaosyl ceramide (Gb_5); globotetraosyl ceramide (Gb_4); globotriaosyl ceramide (Gb_3); lactosyl ceramide (LacCer); type 1 galactosyl ceramide (GalCer1); and glucosyl ceramide (GlcCer)} (3 μg) were separated on 7 mm lanes on ten silica gel plates (Macherey-Nagel, Polygram SIL G/UV_{254}) with a 50 ml mobile phase of 14 : 6 : 1 (chloroform / methanol / water (0.88% KCl), v/v/v). The plates were dried and a reference plate was exposed to iodine vapour to reveal the positions of the lipids. Five plates were blocked for 1 h with 1% bovine serum albumin (BSA) in tris-buffered saline (TBS) (50 mM Tris, 154 mM NaCl, pH 7.5). These plates were then incubated for 1 h with either: AP-conjugated goat anti-mouse (AP-GAM) (γ-chain specific) (1/1000 in 1% BSA in TBS); HRP-conjugated goat anti-mouse (HRP-GAM) (γ-chain specific) (1/1000 in 1% BSA in TBS); AP-conjugated anti-digoxigenin (AP-AD) (Fab fragment) (1/1000 in 1% BSA in TBS); AP-conjugated anti-fluorescein (AP-AF) (Fab fragment) (1/1000 in 1% BSA in TBS); or AP-conjugated streptavidin (AP-SA) (2 μg/ml in 1% BSA in TBS) [all APs were calf intestinal AP, and therefore did not differ in their quality and performance]. The remaining four plates were coated with polyisobutylmethacrylate (PIBM) plastisizer solution (Sigma-Aldrich; 0.5% in 5 : 8 (chloroform / hexane, v/v)) according to Mamelak et al. (2001). These plates were then blocked and incubated with either AP-GAM, AP-AD, AP-AF, or AP-SA as above. The entire procedure (minus development of a reference plate and incubation of lipids with HRP-GAM) was then repeated with TTBS buffer (0.1% Tween 20, 50 mM Tris, 154 mM NaCl, pH 7.5). Prior to development, all plates were washed 5× for 5 min in TBS. AP systems were developed with an AP conjugate substrate kit (Bio-Rad, Hercules, CA, USA) and the HRP system was developed by the addition of one volume of 4-chloro-1-naphthol solution (3 mg/ml in methanol) with 5 volumes of TBS and 1 μl of 30% H_2O_2 per 2 ml of solution. All steps were performed at room temperature.

As compared to staining by iodine vapour, all sphingolipids and phospholipids stained positively in AP-GAM, AP-AD, AP-AF and AP-SA systems, whereas no staining occurred with HRP-GAM (Fig. 1A–F). The positive reaction in AP systems was, however, reduced by treating TLC plates with PIBM (Fig. 1G–J) prior to conjugate addition. When Tween 20 was added to the TBS, binding to LacCer was retained, whereas binding to all other lipids was abolished (Fig. 2A–D). LacCer binding was also detected on plates treated with PIBM (Fig. 2E–H).

The negative result for HRP-GAM indicated that the binding observed with AP-GAM, AP-AD and AP-AF

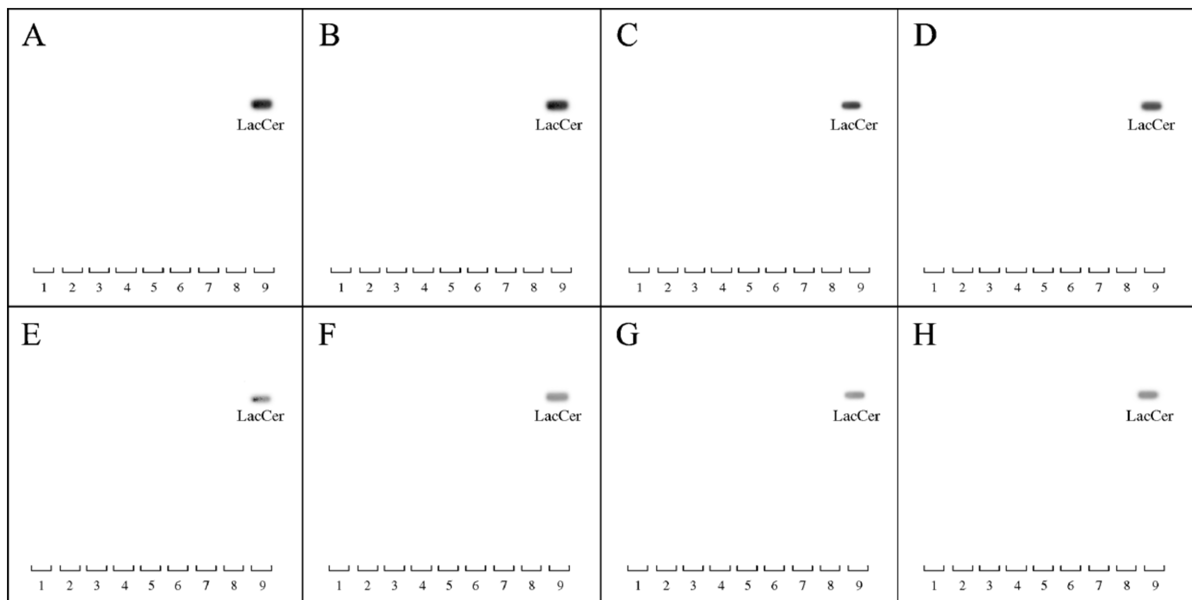

Fig. 2. Effect of Tween 20 on overlays of enzyme conjugates onto phospholipids and sphingolipids. Lanes 1–8 = PE, PS, PI, PC, SM, SGC, GM2, GalCer2, respectively; lane 9 = $Gb_5 + Gb_4 + Gb_3$ + LacCer + GalCer1 + GlcCer. A = AP-GAM overlay; B = AP-AD overlay; C = AP-AF overlay; D = AP-SA overlay; E = AP-GAM overlay with PIBM; F = AP-AD overlay with PIBM; G = AP-AF overlay with PIBM; H = AP-SA overlay with PIBM.

was not associated with the immunoglobulin moiety, but with the AP. This conclusion was verified by the fact that a non-immunoglobulin AP-conjugate, AP-SA, exhibited an identical phospholipid/sphingolipid binding profile. This also indicated that the binding was not due to some aspect of the chemical linkage between AP and conjugate since the AP-IgGs used in this study were made with glutaraldehyde (a procedure that can result in sticky conjugates) whereas the AP-SA was made using a heterofunctional cross-linker (maleimidohexanoic NHS ester and *S*-acetyl thioacetic NHS ester). Furthermore, treatment with PIBM (in TBS) reduced AP-conjugate binding. PIBM was originally designed to disrupt non-selective hydrophobic interactions and reorient lipids on silica to optimize ligand binding to the polar head group (Magnani et al., 1980). Reduced binding due to PIBM, therefore, supports the notion of phospholipid/sphingolipid-AP binding independent of IgG, and likely due to hydrophobic lipid interactions.

Phospholipid/sphingolipid binding by AP-conjugates as a result of non-selective hydrophobic interactions was not unexpected, given the membrane bound location of native AP. While reduced phospholipid/sphingolipid binding due to the addition of Tween 20 supports this notion, the basis for retention of binding to LacCer is not clear. The fact that LacCer binding remains following treatment with PIBM suggests specificity. LacCer has recently been implicated in the regulation of cell signaling and proliferation (Bhunia et al., 1996), while placental AP is targeted to signal transduction hot spots termed 'lipid rafts' in supported lipid bilayers (Saslowsky et al., 2002). The question of a functional physiological connection was beyond the scope of the current study, but certainly warrants investigation.

Hydrophobic-based AP interactions with phospholipids and sphingolipids may be a useful feature for the production of cross-linked AP-lipid conjugates in vitro (e.g. biotinylated lipids bound to AP-SA (Blixt et al., 2003)); however they complicate the use of AP-conjugates for immunological detection in biological samples. PIBM and Tween 20 should always be used for AP based TLC antibody overlay. However, we urge caution in the use of AP-conjugates in immunohistochemistry and in situ hybridization. HRP systems should be used whenever possible, when not possible Tween 20 should be employed and the AP-conjugate should always be first singly overlayed to control for non-selective phospholipid/sphingolipid and/or selective LacCer binding.

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