Glucagon-like peptide-1 (GLP-1)-based diabetes therapy is increasingly used in the treatment of type 2 diabetes. Two GLP-1 analogs, liraglutide and exenatide, have been approved for clinical use and act as agonists of the GLP-1 receptor (GLP-1R), a member of the glucagon receptor family of G protein-coupled receptors (GPCRs) (1). Also, the clinical effect of Dipeptidyl Peptidase-4 (DPP-4) inhibitors is at least partially resulting from an increased level of active GLP-1 (2). The unambiguous identification of GLP-1R-expressing cells in humans and animal species constitutes critically important knowledge for fully understanding the pharmacological effects of GLP-1R agonists, both those associated with the documented clinical benefits as well as any potential undesired effects of treatment.

The GLP-1R was cloned in 1992 (3). Preceding that, the receptor was identified in numerous cell types and organs, most obviously the pancreatic β-cell but also in rat lung and brain by studies using either ligand binding or functional assays (4, 5). After the receptor was cloned, RT-PCR and in situ hybridization were used to report tissue distribution, and the receptor was located in the intestine, stomach, kidney, lungs, heart, and brain (6, 7). Later studies have used Western blotting and immunohistochemistry to identify the exact cellular localization of the receptor. Using such techniques, the receptor has been identified in mouse cardiomyocytes and smooth muscle cells in the kidney (8, 9). Human GLP-1R expression has been mapped by in situ ligand binding across a wide range of both normal and tumor tissues (10).

In this issue of Endocrinology, Panjwani et al. (11) reports new knowledge about the mechanism of action of GLP-1 analogs in atherosclerosis and hepatic steatosis and challenges some GLP-1R expression data in macrophages and hepatocytes that have previously been published. When Panjwani et al. (11) could not identify GLP-1R mRNA on macrophages or isolated hepatocytes despite previous reports of such localization, they did a careful Western blotting characterization of three commercially available polyclonal antibodies (PAbS) against the GLP-1R that are commonly used for Western blotting. They used mouse wild-type lung tissue that has a very high expression of GLP-1R as a positive control and lung tissue from GLP-1R−/− mice as a negative control. They also employed a very thorough procedure with immunoprecipitation in an attempt to increase the sensitivity of the analysis but found no GLP-1R-specific expression. The authors conclude that multiple commercially available GLP-1R antibodies do not detect authentic GLP-1R protein, even using optimally enhanced methods. This technical difficulty at first glance seems to have little bearing on aspects of GLP-1 biology unrelated to the scope of the article; however, on further consideration, it brings into focus a potentially serious problem with the validity of any GLP-1R expression data generated through the use of antibodies.

Adding to the complexity, for some GLP-1R-containing organs, markedly different GLP-1R expression patterns have been reported between human and rodent species (10, 12). Rodent thyroid C-cells express much higher levels of GLP-1R than primates (10, 12), and there may be an important species difference, where the GLP-1R in the rodent thyroid is important for the high bone turnover in those species but less important in humans (13, 14). In the lung, the number of GLP-1R-expressing cells and the receptor density is much lower.
lower in humans than in rodent species (10), but the role and importance of lung GLP-1R is relatively unexplored.

Three different histotechniques are available for mapping the identity of cells expressing a receptor like the GLP-1R. First, in situ hybridization is the method of choice for detecting mRNA in tissues; however, for a low-abundance target like the GLP-1R, only methods depending on the use of radioactively labeled probes will provide the required sensitivity, and unfortunately, the final autoradiography step severely limits the resolution of the output signal. A similar problem exists for in situ ligand binding (ISLB) with radioactively labeled ligand, although this method has been highly useful for detecting a functional GLP-1R in cell types that can be identified despite the limited resolution (12, 15). The third method, and the one most often employed to map GLP-1R localization by researchers in the field, is immunohistochemistry (IHC) with anti-GLP-1R antibodies. IHC offers the best resolution and allows unambiguous cell identification; however, for GPCRs, it is notoriously difficult, and a high focus on validation of antibodies and protocols is imperative. Further adding to the difficulty of generating reliable GPCR protein expression data, the normal physiological cell density of many GPCRs can be very low.

In one issue of the journal Naunyn-Schmiedeberg's Archives of Pharmacology, seven groups of investigators reported protein expression data for a variety of GPCR family members as measured by the use of antibodies, and summarizing these studies, a lack of specificity of commercial antibodies was shown to be the rule rather than the exception (16–21). The antibodies studied were directed against different adrenergic, muscarinic, dopamine, acetylcholine, and galanin receptors. A total of 49 antisera against 19 receptors were tested, in many cases using multiple antibodies, and none of them were found to be selective. An accompanying editorial advocated the rigorous use of a fixed set of four validation criteria for any anti-GPCR antibody (22). The first of these tests is the absence of reactivity in tissues from knockout animals, and the true strength of this control is well illustrated by the study by Panjwani et al. (11) in this issue of Endocrinology. Another validation test criterion suggested is the use of several antibodies raised against multiple distinct epitopes of the receptor and showing similar reactivity. In practice, and certainly in the studies recapitulated in the above mentioned editorial (22), it is extremely difficult to generate, identify, and validate two or more antibodies against different epitopes on the same GPCR.

When the aim is to study expression of a given GPCR in human tissues, the use of the mouse knockout tissue control unfortunately is not always relevant, because anti-GPCR antibody specificity may not be maintained across rodent and nonrodent species. In IHC studies of GLP-1R localization in human and monkey tissues, we used the following two-step procedure to identify and validate anti-GLP-1R antibodies that specifically stain GLP-1R in paraffin-embedded tissue samples. In step 1, each anti-GLP-1R antibody was tested for its ability to specifically detect GLP-1R in transfected cells overexpressing human full-length GLP-1R, using the exact same IHC protocol as in subsequent studies of human tissue material. If the study included formalin-fixed and paraffin-embedded human tissue samples, then the GLP-1R-transfected cells were also formalin-fixed and paraffin-embedded. Antibodies that passed this first validation step were further tested in step 2, where, for each organ studied, an identical distribution of signals for IHC and for ISLB with [125I]GLP-1 for functional GLP-1R must be demonstrated. Using this approach, we tested four commercially available rabbit PAbs that were all recommended by the suppliers for human GLP-1R IHC use on paraffin-embedded material, but with little or no data to validate this application. The antibodies tested were LS-A1205 (LifeSpan Biosciences, Inc. (Seattle, US)), raised against a synthetic 16-amino-acid peptide from the N-terminal extracellular domain of the human GLP-1R; AP-31362 (Acris Antibodies GmbH, (Herford, GER)), raised against a synthetic peptide from the human GLP-1R; AP-23801 (Acris Antibodies GmbH (Herford, GER)), raised against a synthetic peptide from the C-terminal domain of the human GLP-1R; and ab39072 (Abcam plc (Cambridge, UK)), raised against a synthetic peptide derived from within residues 250–350 of the human GLP-1R, and used in the GLP-1 literature (23). For all antibodies, a cytoplasmic staining of cells in human islets of Langerhans is presented by the suppliers to support the specificity of GLP-1R in IHC protocols; however, pancreas islets are known to frequently give rise to false-positive staining in IHC studies (24). In addition, the absence of a clear membrane-associated staining on β-cells in any of the micrographs also does not support specificity for the GLP-1R, given the well-established fact that this and other GPCRs are membrane-spanning molecules. Due to discontinuation of supply, we were not able to include the PAb LS-A1206, also recommended for IHC on paraffin-embedded human material, and used in other studies (25, 26).

Figure 1 shows a typical result of this first validation step, i.e. the ability of antibodies to react with human GLP-1R-transfected cells and not with nontransfected...
cells. All of the four commercial antibodies recommended for IHC reacted with equal intensity with both GLP-1R and untransfected cells and, when further diluted to a concentration where there was negligible staining of untransfected cells, did not react with GLP-1R-transfected cells. Human GLP-1R-transfected cells typically express 100-fold higher levels of receptors than cells with endogenous expression of the receptor (27), and based on this, we find it unlikely that protocol optimization could lead to the generation of specific immunoreactivity signals in human paraffin-embedded samples using any of the four commercial antibodies. A monoclonal anti-GLP-1R antibody (MAb 3F52) generated by us and selected for its reactivity with monkey GLP-1R in paraffin-embedded tissues gave a more specific staining pattern. MAb 3F52 was raised in GLP-1R−/− mice against the human GLP-1R extracellular domain. With this antibody, a clear staining was seen of GLP-1R-transfected cell lines with no reactivity to non-transfected cells, and the staining was both cytoplasmic and membrane-associated. Moreover, when MAb 3F52 was further tested on human and monkey pancreas samples as part of step 2 of our IHC validation, a pronounced and membrane-associated staining of β-cells was observed as shown in Fig. 2 for a rhesus macaque monkey pancreas. Several other organs were included in the analysis, including the entire pancreas, heart, and

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**FIG. 1.** IHC staining with four commercial PAbs and one new monoclonal antibody (MAb) against the human GLP-1R expressed in BHK cells. The IHC was performed with the Dako EnVision™ FLEX, high pH system as recommended by the supplier (DAKO A/S, Glostrup, DK). In brief, formalin-fixed and paraffin-embedded sections of human GLP-1R-transfected and untransfected BHK cells were dewaxed and rehydrated to H2O. Sections were microwave treated in Tris-EGTA buffer (pH 9.0) and then treated with 1% H2O2 in Tris-buffered saline and processed according to the protocol recommended by the supplier. The antibodies were all tested in two different concentrations, and a polyclonal and a monoclonal antibody against another antigen were included as extra negative (Neg.) controls.
kidney, and identical staining patterns were found with IHC and ISLB (full manuscript in preparation).

Given the technical challenges of obtaining reliable GLP-1R protein expression data, how do we obtain rock-solid data of the cellular identity of GLP-1R-expressing cells in vivo? As clearly demonstrated by Panjwani and colleagues (11) in this issue, if cell identification is based on the use of antibodies, there is no way around employing very extensive validation criteria. The way forward is for researchers in the field to apply and publish the results of such rigorous validation tests, so that all truly validated selective antibodies available can be identified and broadly applied. Combining IHC using validated antibodies with ISLB is a strong documentation of the presence of functional GLP-1Rs, and the use of double-labeling IHC with antibodies against specific cell markers further validates the findings. As an alternative to radiolabeled ligands, the use of fluorescence-labeled GLP-1 analogs in vivo or ex vivo also appears very promising. Reiner et al. (28) reported in the Proceedings of the National Academy of Sciences of the United States of America in 2011 the generation of fluorophore-labeled exendin-4 and used it for high-resolution imaging; this and related approaches will likely have the specificity and sensitivity required for cell identification. To determine whether and where the GLP-1R is expressed at low levels not detectable by validated IHC or imaging methods, an attractive approach could be the combined use of laser capture microdissection with quantitative PCR (29).

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Address all correspondence and requests for reprints to: Lotte Bjerre Knudsen, Novo Nordisk, Novo Nordisk Park, DK-2760 Maaloev, Denmark. E-mail: lbkn@novonordisk.com.

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