Volume 318, number 2, 181–185 FEBS 12145 March 1993

Interactions of calreticulin with proteins of the endoplasmic and sarcoplasmic reticulum membranes

Kimberly Burns and Marek Michalak

Cardiovascular Disease Research Group and Department of Biochemistry, University of Alberta, Edmonton, Alta. T6G 2S2, Canada.

Received 20 October 1992; revised version received 12 January 1993

The ability of [^{125}I]calreticulin to bind to membrane fractions isolated from different muscle and non-muscle tissues was examined by a protein overlay technique. Specific [^{125}I]calreticulin binding proteins were detected in rat liver smooth and rough endoplasmic reticulum and Golgi, in canine pancreatic microsomes, and in rabbit skeletal muscle sarcoplasmic reticulum. These proteins were confined only to membranes that contain calreticulin; they were not found in rat liver mitochondria or cytosol. [^{125}I]Calreticulin binds to a 50-kDa protein and a number of lower M_r (20,000–38,000) endoplasmic reticulum membrane proteins and to 30-kDa protein in skeletal muscle sarcoplasmic reticulum. Full-length calreticulin and the carboxyl-terminal region (*C-domain*) of the protein both competed with [^{125}I]calreticulin for binding to the membrane proteins. Binding of [^{125}I]calreticulin to pancreatic microsomes was also partially inhibited by the *N-domain* and to a lesser extent by the *P-domain* of the protein. We conclude that calreticulin interacts with the endoplasmic reticulum membrane proteins mainly through its carboxyl-terminal domain and that the endoplasmic and sarcoplasmic reticulum membranes may contain different calreticulin binding proteins.

Calreticulin; Calcium binding protein; Endoplasmic reticulum; Sarcoplasmic reticulum; Protein overlay

1. INTRODUCTION

Calreticulin is a major Ca^{2+}-binding, peripheral membrane protein of the lumen of the ER (for review see [1]). Calreticulin is also localized to the nuclear envelope in a variety of cells and to the nucleus in L6 cells [2]. Based on the ability of calreticulin to bind >25 mol of Ca^{2+}/mol of protein [3,4], it has been proposed that calreticulin may play a Ca^{2+} storage role in the ER [1]. This is analogous to calsequestrin, the main Ca^{2+} storage protein of skeletal muscle SR [5]. Recent reports indicate that calreticulin may interact with different cellular proteins and thus have more than one cellular function [1].

In this study, to determine if calreticulin associates with other components of the ER membranes, we have employed a protein overlay (ligand blotting) technique. Using this technique we have detected proteins that interact with calreticulin in rat liver ER, Golgi membranes and the nucleus, in canine pancreatic microsomes, and in rabbit skeletal muscle SR. Interaction with these proteins is mediated mainly through the *C-domain* of calreticulin.

Correspondence address: M. Michalak, 424 Heritage Medical Research Center, Cardiovascular Disease Research Group, Department of Biochemistry, University of Alberta, Alberta, Canada T6G 2S2. Fax: (1) (403) 492-3383.

Abbreviations: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. EXPERIMENTAL PROCEDURES

Pancreatic microsomes were isolated by the procedure described by Walter and Blobel [6]. Smooth and rough ER, Golgi, nuclei and the cytosolic fractions were isolated from rat liver as described by Croze and Morre [7]. SR membrane fractions were isolated from rabbit skeletal muscle [8]. Initial tissue homogenizations and subsequent membrane fractionations were performed in the presence of a mixture of the protease inhibitors [9]. Isolated membrane vesicles were frozen in liquid N_2 and stored at −85°C. Protein was determined by the method of Lowry et al. [10].

Calreticulin was purified from dog pancreas by a selective ammonium sulfate precipitation method [11]. Recombinant full-length calreticulin and the domains of calreticulin were expressed in *E. coli* as glutathione *S*-transferase (GST) fusion proteins and purified [4,11]. Iodination of the native and recombinant calreticulin was carried out using Iodo-Gen (Pierce Chemical Co.) as described by Beisiegel et al. [12].

SDS-PAGE (10% acrylamide) was carried out as described by Laemmli [13]. After gel electrophoresis, proteins were transferred electrophoretically onto nitrocellulose membrane according to the method of Towbin et al. [14]. Transferred proteins were visualized with Ponceau-red, which did not affect [^{125}I]calreticulin binding to the blots. Before incubation with [^{125}I]calreticulin, the nitrocellulose blots were preincubated for 1 h in blocking buffer containing 20 mM Tris, 150 mM KCl, 5% bovine serum albumin and either 2 mM $ZnSO_4$, 5 mM $CaCl_2$ or 5 mM EGTA. [^{125}I]Calreticulin (10^6 cpm/ml) was added to freshly prepared blocking buffer and incubated with the nitrocellulose strips for 2 h at room temperature. The blots were rinsed 3 times with blocking buffer, followed by two 30 min washes in blocking buffer. In competition experiments unlabeled proteins were added at a concentration of 1 mg/ml. Dried nitrocellulose filters were exposed to Kodak XAR-5 film. Standards were Bio-Rad low range molecular weight proteins or Bio-Rad prestained markers.

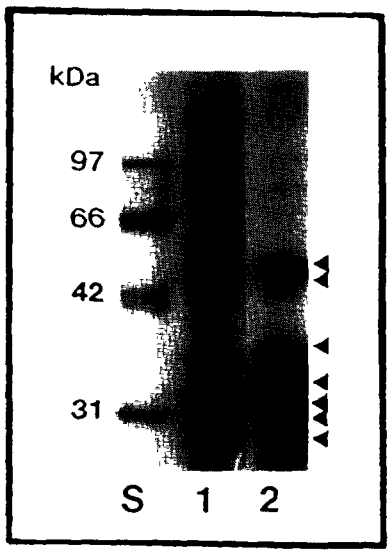

Fig. 1. SDS-PAGE and binding of ^{125}I-labeled calreticulin to pancreatic membrane proteins. Pancreatic microsomal membranes were subjected to SDS-PAGE, transferred to nitrocellulose and analyzed by ligand blotting with ^{125}I-labeled calreticulin as described under the Experimental Procedures. Lane S, Bio-Rad low range molecular weight markers; lane 1, Coomassie blue staining of pancreatic microsomes; lane 2, autoradiographs of [^{125}I]calreticulin binding proteins in pancreatic microsomes. Lane 1, 30 μg of protein, lane 2, 80 μg of protein. Arrowheads indicate calreticulin binding proteins.

3. RESULTS

Seven major proteins were consistently detected that interacted with [^{125}I]calreticulin in pancreatic membranes; a protein doublet at 50- and 52-kDa, and a series of lower molecular weight proteins ranging from 38,000 to 20,000 (Fig. 1, lanes 2, arrowheads). Though calreticulin is a Ca^{2+} and Zn^{2+} binding protein [1], the binding of [^{125}I]calreticulin to the microsomal proteins on the nitrocellulose blots was neither Ca^{2+} nor Zn^{2+} dependent (data not shown). Excess of unlabeled calreticulin competed with [^{125}I]calreticulin for binding to the proteins of the pancreatic microsomes (Fig. 2).

To establish which region of calreticulin was responsible for the observed interactions, the *N-*, *P-* and *C-domains* [1] were used in competition experiments. The GST-*C-domain*, but not GST alone, efficiently competed with [^{125}I]calreticulin for binding to pancreatic microsomal proteins (Fig. 3, lanes D and E). The GST-*N-domain* partially inhibited [^{125}I]calreticulin binding to the membrane proteins (Fig. 3, lane B). The GST-*P-domain* had little effect on [^{125}I]calreticulin binding and only partially inhibited [^{125}I]calreticulin binding to the 50-kDa doublet protein band (Fig. 3, lane C). Therefore, calreticulin appears to interact with these proteins mainly via its carboxyl-terminal region (*C-domain*).

Calreticulin has been localized to the ER membranes, nuclear envelope and in L6 cells to the nucleus [2,15]. Fig. 4A shows that [^{125}I]calreticulin interacts with a similar set of proteins in rat liver microsomes (lane 1), liver smooth and rough ER (lanes 2 and 3) and Golgi (lane 4). In contrast, in the nucleus three proteins of 35-kDa, 33-kDa and 30-kDa were detected (Fig. 4A, lane 8). A major difference between the pattern of calreticulin binding proteins in smooth and rough ER was that in the former the 52-kDa protein band was not labeled (Fig. 4A, lane 2). It is difficult to establish if the labeling pattern found in smooth ER membranes corresponds to

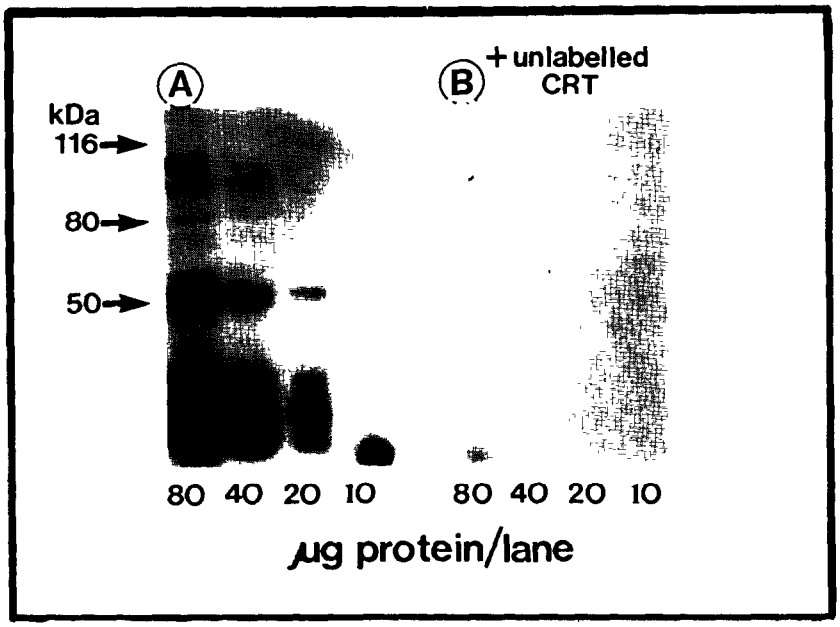

Fig. 2. Inhibition of binding of [^{125}I]calreticulin to pancreatic microsomes by unlabeled calreticulin. Different amounts of pancreatic microsomal proteins were subjected to SDS-PAGE followed by transfer to nitrocellulose and by an overlay with a ^{125}I-labeled calreticulin in the absence (A) or presence (B) of unlabeled calreticulin (1 mg/ml). The positions of Bio-Rad prestained molecular mass standards are indicated.

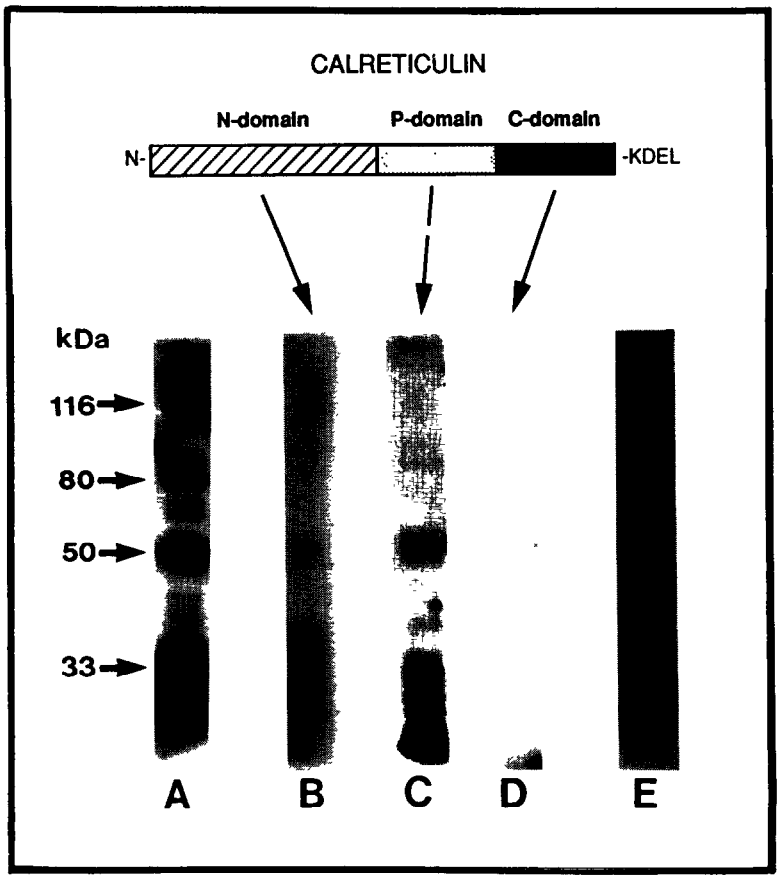

Fig. 3. *C-domain* of calreticulin inhibits binding of ^{125}I-labeled calreticulin to pancreatic microsomes. Pancreatic microsomes (80 μg of protein) were subjected to SDS-PAGE followed by transfer to nitrocellulose. Lane A, overlay with a ^{125}I-labeled calreticulin; lanes B, C and D [^{125}I]calreticulin overlay were carried out in the presence of 1 mg/ml of the GST-*N-domain*, GST-*P-domain* and GST-*C-domain*, respectively. Lane E, [^{125}I]calreticulin overlay in the presence of GST (1 mg/ml). The positions of Bio-Rad prestained molecular mass standards are indicated.

the true smooth ER membrane or contaminations derived from the rough ER. The smooth ER fraction used in this study consists of a mixture of smooth ER membranes and rough ER membrane from which ribosomes have become dissociated during the isolation [7]. Fig. 4A, lanes 5–7 also shows binding of [^{125}I]calreticulin to skeletal muscle SR membrane proteins. A protein band of 30-kDa was the major [^{125}I]calreticulin binding protein in the heavy and light SR membranes. Smaller quantities of a 90-kDa and 50-kDa protein also bound [^{125}I]calreticulin in these SR membranes (Fig. 4A, lanes 5–7).

To establish the specificity of our ligand binding method we also tested subcellular fractions that are known not to contain any immunologically detectable calreticulin, i.e. mitochondria and cytosol [2,15]. Fig. 4B shows that [^{125}I]calreticulin does not bind to mitochondrial (Fig. 4B, lane 2) or cytosolic (Fig. 4B, lane 3) proteins isolated from rat liver. In addition, there was only one protein (~ 33-kDa) detected when a large amount (>200 μg protein/lane) of *E. coli* cellular extract was tested for [^{125}I]calreticulin binding (Fig. 4B, lane 5). [^{125}I]Calreticulin did not bind to the purified, unlabeled calreticulin (Fig. 4B, lane 4).

4. DISCUSSION

Using a protein overlay technique we have detected a set of proteins that bind [^{125}I]calreticulin in rat liver ER, Golgi, canine pancreatic microsomes (predominantly rough ER), nuclei and in rabbit skeletal muscle SR. In non-muscle ER membranes, native or recombinant [^{125}I]calreticulin binds to a 50- and 52-kDa protein band doublet and a number of lower M_r (38–20-kDa) proteins. In contrast, muscle SR membranes contain only one major calreticulin binding protein of M_r ~ 30 000. Full-length calreticulin competed with [^{125}I]calreticulin for binding to the membrane proteins. We have shown here that the calreticulin binding proteins were confined only to the membranes that contain calreticulin; they were not found in rat liver mitochondria or cytosol, and they were identified only in small amounts in the skeletal muscle SR.

Ligand binding (blotting) method is a simple yet pow-

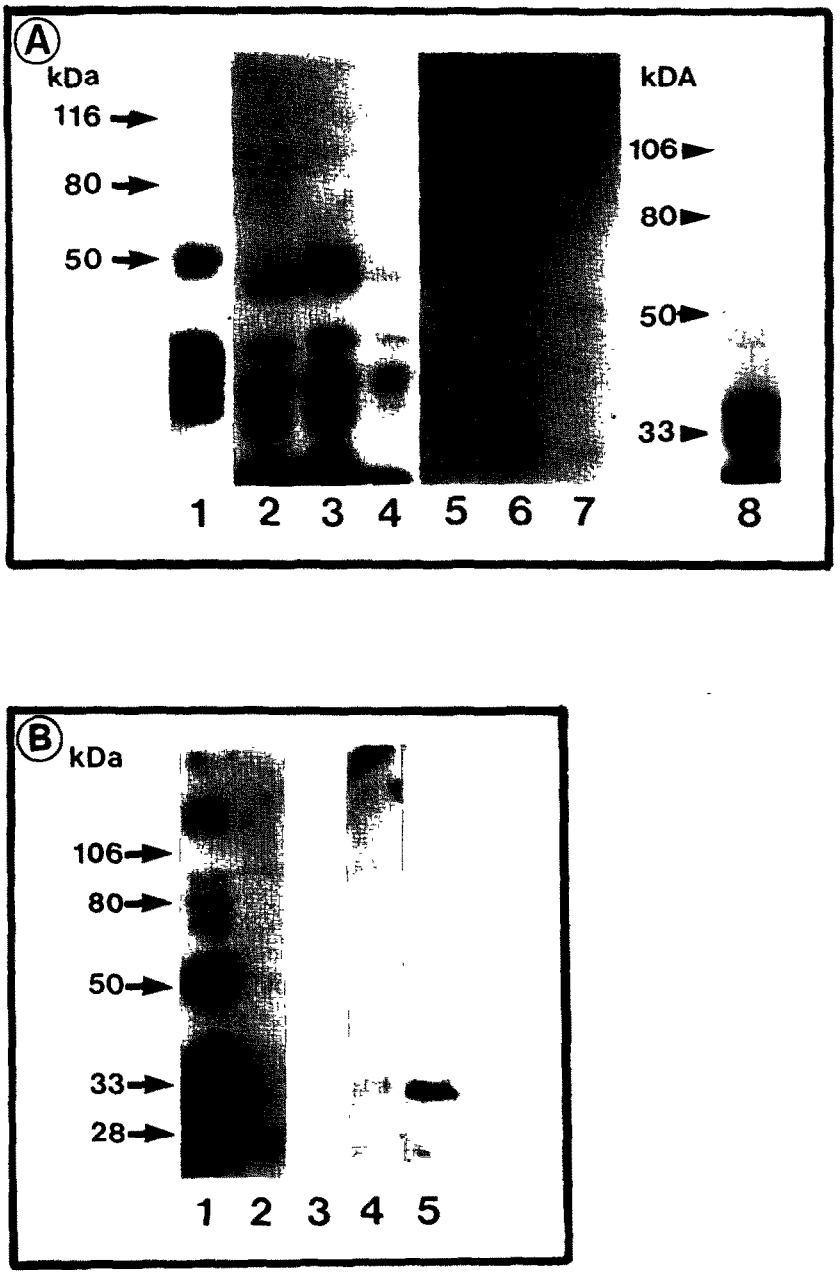

Fig. 4. Binding of ^{125}I-labeled calreticulin to different membrane fractions. (A) Lane 1, rat liver microsomes; lane 2, rat liver smooth ER; lane 3, rat liver rough ER; lane 4, rat liver Golgi membranes; lane 5, rabbit skeletal muscle light SR membrane; lane 6, rabbit skeletal muscle heavy SR membrane; lane 7, rabbit skeletal muscle microsomes, lane 8, rat liver nuclei. (B) Lane 1, pancreatic microsomes; lane 2, rat liver mitochondria; lane 3, rat liver cytosolic proteins (100,000 × *g* supernatant); lane 4, purified, recombinant calreticulin; lane 5, *E. coli* cellular extract. The positions of Bio-Rad prestained and low range molecular mass standards are indicated.

erful technique to identify protein–protein interactions It has been used in the past to identify calmodulin binding protein [16], low density lipoprotein receptor [17] and calsequestrin binding proteins [18,19]. Calsequestrin and calreticulin share some basic functional similarities, as both proteins are considered to be Ca^{2+} binding (storage) sites in the lumen of muscle SR and non-muscle ER membranes, respectively [1,5]. Mitchell et al. [19] was first to employ ligand binding (protein overlay) method for the identification of calsequestrin binding protein(s) in cardiac SR membranes. They identified a 26-kDa calsequestrin-binding protein in that membrane system. Recently, Damiani and Margreth [18] have described a set of calsequestrin binding proteins (47-kDa; 31–30-kDa, 29-kDa and 26–25-kDa) in skeletal muscle SR membrane. Calsequestrin interacts with these SR membrane proteins in a Ca^{2+}-dependent manner [18,19]. A 30-kDa calreticulin binding protein

identified in this study in the SR membranes may correspond to the 26-kDa calsequestrin binding protein identified by Mitchell et al. [19].

Calreticulin can be divided into three structurally different regions referred to as *N*-, *P*- and *C-domain* that may perform different functions [1]. For example, the high affinity and the low affinity Ca^{2+} binding sites are localized to the *P-domain* and *C-domain*, respectively [4]. Although the *N*- and *P-domains* may contribute to [^{125}I]calreticulin binding to microsomal proteins, the C-terminal region (*C-domain*) is likely the main region involved in the observed protein–protein interactions. This region of the protein is relatively acidic and has been proposed to exist in an extended conformation [1]. The involvement of the carboxyl-terminal region of calreticulin in protein–protein interactions may be significant in regards to a recent observation. Wada et al. [20] cloned an integral membrane protein of the ER (named calnexin) which shows marked similarity to calreticulin in its amino acid sequence. The highest similarity between the two proteins is confined to the *P*- and *C-domain* of calreticulin. The carboxyl-terminal region of calnexin ('*C-domain*') has been proposed to be exposed to the cytoplasmic site of the ER membranes. We showed that the *C-domain* of calreticulin is involved in protein–protein interactions, suggesting that this region of calnexin may also interact with other proteins. Based on recent observations [21–23] it was proposed that calnexin may function as a molecular chaperone and play a role in the folding and/or assembly of newly synthesized polypeptide chains. It is possible, therefore, that calreticulin, a Ca^{2+} binding (storage) protein, interacts with other cellular proteins and may also play a chaperon-like role in the lumen of the endoplasmic reticulum.

Acknowledgements: This work was supported by grants from the Medical Research Council of Canada and the Heart and Stroke Foundation of Alberta. We would like to thank Dr. J. Vance (University of Alberta) for a generous gift of the purified rat liver membrane fractions and Dr. D.L. Barber for his invaluable help in performing iodination experiments. We are grateful to C. Shemanko for helpful discussions and for critical reading of the manuscript. The excellent technical assistance of Ms. B. Petryk is greatly appreciated. K.B. is a recipient of a Studentship from the Alberta Heritage Foundation for Medical Research. M.M. is a Medical Research Council Scientist and a Scholar of the Alberta Heritage Foundation for Medical Research.

REFERENCES

[1] Michalak, M., Milner, R.E., Burns, K. and Opas, M. (1992) Biochem. J. 285, 681–692.
[2] Opas, M., Dziak, E., Fliegel, L. and Michalak, M. (1991) J. Cell. Physiol. 149, 160–171.
[3] Ostwald, T.J. and MacLennan, D.H. (1974) J. Biol. Chem. 249, 974–979.
[4] Baksh, S. and Michalak, M. (1991) J. Biol. Chem. 266, 21458–21465.
[5] MacLennan, D.H., Campbell, K.P. and Reithmeier, R.A.F. (1983) in: Calcium and Cell Function (Cheng, W.Y. ed.) vol. 4, pp. 151–173, Academic Press, New York.
[6] Walter, P. and Blobel, G. (1983) Methods Enzymol. 96, 682–691.
[7] Croze, E.M. and Morre, D.J. (1984) J. Cell Physiol. 119, 46–57.
[8] Meissner, G. (1984) J. Biol. Chem. 259, 2365–2374.
[9] Milner, R.E., Baksh, S., Shemanko, C., Carpenter, M.R., Smillie, L., Vance, J.E., Opas, M. and Michalak, M. (1991) J. Biol. Chem. 266, 7155–7165.
[10] Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Baksh, S., Burns, K., Busaan, J. and Michalak, M. (1992) Prot. Exp. Pur. 3, 322–331.
[12] Beisiegel, U., Schneider, W.J., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1981) J. Biol. Chem. 256, 11923–11931.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[15] Michalak, M., Baksh, S. and Opas, M. (1991) Exp. Cell Res. 197, 91–99.
[16] Bachs, O. and Carafoli, E. (1987) J. Biol. Chem. 262, 10786–10790.
[17] Daniel, T.O., Schneider, W.J., Goldstein, J.L. and Brown, M.S. (1983) J. Biol. Chem. 258, 4606–4611.
[18] Damiani, E. and Margreth, A. (1990) Biochem. Biophys. Res. Commun. 172, 1253–1259.
[19] Mitchell, R.D., Simmerman, H.K.B. and Jones, L.R. (1988) J. Biol. Chem. 263, 1376–1381.
[20] Wada, I., Rindress, D., Cameron, P.H., Ou, W.-J., Doherty II, J.J., Louvard, D., Bell, A.W., Dignard, D., Thomas, D.Y. and Bergeron, J.J.M. (1991) J. Biol. Chem. 266, 19599–16610.
[21] Ahluwalia, N., Bergeron, J.J.M., Wada, I., Degen, E. and Williams, D.B. (1992) J. Biol. Chem. 267, 10914–10918.
[22] Hochstenbach, F., David, V., Watkins, S. and Brenner, M.B. (1992) Proc. Natl. Acad. Sci. USA 89, 4734–4738.
[23] Galvin, K., Krishna, S., Ponchel, F., Frohlich, M., Cummings, D.E., Carlson, R., Wands, J.R., Isselbacher, K.J., Pillai, S. and Ozturk, M. (1992) Proc. Natl. Acad. Sci. USA 89, 8452–8456.