Cyclic AMP-dependent Protein Kinase Binding to A-kinase Anchoring Proteins in Living Cells by Fluorescence Resonance Energy Transfer of Green Fluorescent Protein Fusion Proteins*

(Received for publication, July 21, 1999)

Mary Louise Ruehr‡, Daniel R. Zakhary‡§, Derek S. Damron¶, and Meredith Bond‡§

From the ‡Department of Molecular Cardiology, Lerner Research Institute, ‡Center for Anesthesiology Research, Cleveland Clinic Foundation, and §Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44195

A-kinase anchoring proteins tether cAMP-dependent protein kinase (PKA) to specific subcellular locations. The purpose of this study was to use fluorescence resonance energy transfer to monitor binding events in living cells between the type II regulatory subunit of PKA (RII) and the RII-binding domain of the human thyroid RII anchoring protein (Ht31), a peptide containing the PKA-binding domain of an A-kinase anchoring protein. RII was linked to enhanced cyan fluorescent protein (ECFP), Ht31 was linked to enhanced yellow fluorescent protein (EYFP), and these constructs were coexpressed in Chinese hamster ovary cells. Upon excitation of the donor fluorophore, Ht31-ECFP, an increase in emission of the acceptor fluorophore, RII-EYFP, and a decrease in emission from Ht31-ECFP were observed. The emission ratio (acceptor/donor) was increased 2-fold (p < 0.05) in cells expressing Ht31-ECFP and RII-EYFP compared with cells expressing Ht31P-EYFP, the inactive form of Ht31, and RII-EYFP. These results provide the first in vivo demonstration of RII/Ht31 interaction in living cells and confirm previous in vitro findings of RII/Ht31 binding. Using surface plasmon resonance, we also showed that the green fluorescent protein tags did not significantly alter the binding of Ht31 to RII. Thus, fluorescence resonance energy transfer can be used to directly monitor protein-protein interactions of the PKA signaling pathway in living cells.

There is now increasing evidence for clustering and compartmentalization of signaling enzymes with their activators and target proteins. This targeting of enzymes with their substrates may promote specificity of signaling events (1–3). To better understand the physiological role of scaffolding proteins, such as A-kinase anchoring proteins (AKAPs), which target cyclic AMP-dependent protein kinase (PKA) (2, 4), it is essential to monitor in real time the interactions between the components of this signaling complex in living cells. However, to date such measurements have not been reported for the PKA pathway.

AKAPs and PKA co-localize in cells. This was first shown by the redistribution of the type II regulatory (RII) and the catalytic subunits of PKA from the cytosolic to the particulate fraction following overexpression of AKAP75 in HEK293 cells (5). In studies in HEK293 cells, S-AKAP84 targeted RII to mitochondria (6). In rat cardiac myocytes, endogenous AKAP100 co-localized with RII in the region of the junctional sarcoplasmic reticulum/transverse tubule (7). A functional role of AKAP targeting of PKA is further indicated by studies in which Ht31 peptide, a competitive inhibitor of AKAP/PKA binding, was introduced into AKAP-overexpressing cells. For example, transfection of RINm5F pancreatic beta cells with Ht31 caused redistribution of RII from a perinuclear to a diffuse cytoplasmic localization and prevented cAMP-dependent insulin secretion (8). Similarly, in cardiac myocytes transfected with AKAP79, microinjection of Ht31 impaired the PKA-dependent increase in L-type Ca²⁺ current (9). These studies demonstrate a role for AKAPs in the regulation of PKA distribution and in the specificity of PKA function.

To date, the binding of RII with full-length AKAPs or with the peptide Ht31 has been demonstrated in vitro by RII overlay assay (10–12), by band shift assays on non-denaturing gels (12, 13), by equilibrium dialysis measurements (12, 13), and recently by solution NMR measurements of the N-terminal dimerization domain of RII with Ht31 (14). Equilibrium dialysis measurements (12) and surface plasmon resonance showed that Ht31 binds RII with nanomolar affinity. Although these in vitro approaches provide evidence for high affinity RII/AKAP or RII/Ht31 interactions in vitro, the results presented here extend these studies by measuring real-time interactions between these molecules in living cells.

Fluorescence resonance energy transfer (FRET) was used to analyze the interaction between RII-EYFP and Ht31-ECFP expressed in CHO cells. We used enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) (16) to construct the fusion proteins, RII-EYFP, Ht31-ECFP, and Ht31P-ECFP (the inactive, prolinated Ht31 analog). A significant increase in RII-EYFP fluorescence emission was observed in cells expressing RII-EYFP/Ht31-ECFP compared with control cells expressing RII-EYFP/Ht31P-ECFP thus demonstrating binding of RII to Ht31-ECFP.

* This work was supported by National Institutes of Health Grants RO1 HL62565 (to M. B.), T32 DK07678 and F32 HL10273 (to M. L. R.), and F32 HL07653 (to D. R. Z.) and by a beginning grant-in-aid from the Ohio Valley affiliate of the American Heart Association (to D. S. D.).

‡ To whom correspondence should be addressed: Dept. of Molecular Cardiology NB50, Lerner Research Inst., Cleveland Clinic Foundation, Cleveland, OH 44195. Tel.: 216-444-3734; Fax: 216-444-9263; E-mail: bondm@ccf.org.

¶ The abbreviations used are: AKAP, A-kinase anchoring protein; PKA, cyclic AMP-dependent protein kinase; FRET, fluorescence resonance energy transfer; RII, type II regulatory subunit of PKA; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; Ht31, the RII-binding domain of the human thyroid RII anchoring protein; Ht31P, the inactive prolinated form of the RII-binding domain of the human thyroid RII anchoring protein; GFP, green fluorescent protein; SA, streptavidin; RIIB, biotinylated RII; Ht31P, biotinylated Ht31; Ht31P, biotinylated Ht31; CHO, Chinese hamster ovary.

† Zakhary, D. R., Moravec, C. S., and Bond, M., in press.
ACTII interacts with Ht31, the RII–Ht31P interaction, and its binding domain in live cells.

**EXPERIMENTAL PROCEDURES**

**Construction of Vectors for the Expression of GFP-tagged Proteins—** The cDNAs for RII, Ht31, and Ht31P were gifts from John Scott (Vollum Institute, Howard Hughes Medical Institute, Portland, OR). The construct for Ht31, used in the FRET experiments, encodes residues 418–718 of the human thyroid RII anchoring protein (8). The RII-binding domain, residues 493–515 (12), is included in this peptide. The construct for Ht31 contains residues 418–718 from the same AKAP but with proline substitutions at positions 498 and 507 that prevent Ht31P from binding RII (12). The cDNA encoding ECFP or EYFP (gifts from Roger Tsien, Howard Hughes Medical Institute, University of California, San Diego, CA) was ligated into EGF-P-N1 (CLON-TECH) using AgeI and BsrGI to form pECFP or pEYFP, respectively. The cDNAs for RII, Ht31, or Ht31P were amplified by polymerase chain reaction to remove the stop codon and add restriction enzyme sites for BglII, XhoI, and BamHI. The cDNA encoding Ht31 or Ht31P was excised using BglII and XhoI and ligated into pECFP upstream of ECFP to create pHt31-ECFP or pHt31-EYFP, respectively. The cDNA encoding RII was excised using XhoI and BamHI and ligated into pEYFP downstream of ECFP to create pHt31P-ECFP. Thus, ECFP or EYFP was expressed at the C terminus of each of the proteins of interest. Constructs were verified by DNA sequencing.

**Transfection and Selection of Positive Cells—** CHO cells were stably transfected with pECFP, pEYFP, pHt31-ECFP, or pHt31-EYFP using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s protocol. Cells were subcultured 1:5 into selection media (0.5 mg/ml G418) 48 h posttransfection. Stable transfectants were amplified and sorted by fluorescence-activated cell sorter analysis using excitation wavelengths of 410 nm for ECFP-expressing cells and 480 nm for EYFP-expressing cells. For the FRET experiments, cells stably transfected with Ht31-ECFP or Ht31P-ECFP were plated on 30-mm glass-bottom dishes and then transiently transfected with RII-EYFP using Lipofectin, similar to the methods described by Miyawaki et al. (16) in their FRET study.

**Western Blot Analysis—** Western blot analysis was used to verify that transfected CHO cells expressed the appropriate fusion proteins. Adherent cells were released by trypsin-EDTA treatment, washed with phosphate-buffered saline, pelleted, and resuspended in ice-cold lysis buffer (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10% glycerol, pH 7.4) containing protease inhibitors (30 mM/ml apro- tin, 10 mM leupeptin, 30 mM/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and lysed by using a freeze-thaw method. Proteins from the cell lysate (10 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis using a 15% separating gel and a 4% stacking gel. Molecular mass markers are on the left of the gel. The relative migration of proteins is indicated by the numbers below these markers. Reprinted with permission from the Molecular Biotechnology Core Facility at the Cleveland Clinic Foundation.

**Expression of Fluorescent Fusion Proteins in Transfected CHO Cells—** Western blot analysis was used to verify that transfected CHO cells contained the appropriate fusion proteins. (Fig. 1). The presence of immunoreactive bands confirmed that cells expressed either the 65-kDa Ht31-ECFP or Ht31P-ECFP fusion proteins and/or the 80-kDa RII-EYFP fusion protein. Densitometric scanning of the blots revealed only a 2% difference in expression levels between Ht31-ECFP and Ht31P-ECFP in stably transfected cells (n = 4). Lysate was also

**RESULTS**

**Expression of Fluorescent Fusion Proteins in Transfected CHO Cells—** Western blot analysis was used to verify that transfected CHO cells contained the appropriate fusion proteins (Fig. 1). The presence of immunoreactive bands confirmed that cells expressed either the 65-kDa Ht31-ECFP or Ht31P-ECFP fusion proteins and/or the 80-kDa RII-EYFP fusion protein. Densitometric scanning of the blots revealed only a 2% difference in expression levels between Ht31-ECFP and Ht31P-ECFP in stably transfected cells (n = 4). Lysate was also

**Fluorescence Microscopy—** Cells were plated on glass coverslips to 95% confluency, washed with phosphate-buffered saline, and fixed using ice-cold methanol. Cells containing the RII-EYFP fusion protein were viewed directly using a fluorescein isothiocyanate filter. Immunofluorescent techniques were used to view cells expressing ECFP or an ECFP fusion protein. These cells were fixed, blocked with 0.3% bovine serum albumin in phosphate-buffered saline, and probed with a primary antibody to GFP and a fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch). Coverslips were then mounted onto slides, and cells were viewed using a fluorescein isothiocyanate filter. Although an indirect method was used for viewing ECFP-expressing cells, both methods can identify the location of respective fusion proteins.

**Preparation of Recombinant RIIα and Synthetic Peptides—** Purified recombinant RIIα was prepared as described previously (7). Recombinant RII protein was biotinylated (RIIα) using a BAC-SulfoNHS reaction (Sigma) according to manufacturer’s instructions. Ht31 and Ht31P (residues 493–515 of the full-length protein) were biotinylated at the N terminus (Ht31α, Ht31Pα) to allow detection of RII binding events (17). All peptides were synthesized by the Molecular Biotechnology Core Facility at the Cleveland Clinic Foundation.

**Surface Plasmon Resonance—** Surface plasmon resonance was used to investigate the effects of adding a GPF derivative to the C termini of RII and Ht31. Measurements were taken using the BIAcore 3000 in protein A surface immobilized with an anti-GFP antibody (monoclonal, Roche Molecular Biochemicals) that recognizes both ECFP or EYFP mutants. Densitometry was performed using NIH Image Software to determine differences in expression levels of Ht31 and Ht31P and to determine whether equal expression levels of Ht31 and Ht31P were detected. Flow-rate variation experiments confirmed that both SA surfaces (RIIα and Ht31α) were not limited by mass transport effects. Kinetic constants were calculated by global fitting of the data to a 1:1 Langmuir binding model after subtraction of the control surface, using BIAevaluation software, version 3.0, according to the BIAcore pseudo-first order rate equation as described by Zakharuk et al. (2).

**FRET Analysis of Cells That Contain Ht31-ECFP or Ht31P-ECFP and RII-EYFP—** FRET experiments were performed 48 h after transient transfection of Ht31- or Ht31P-expressing CHO cells with RII-EYFP. Each experiment was performed on 15–20 cells at ~80% confluency. Fluorescence was observed with an Olympus IX-70 inverted fluorescence microscope and recorded using the Felix software imaging system (Photon Technology International). Using this system, excitation was set by a monochromator to the peak excitation wavelength for ECFP, 440 ± 5 nm (using a 455DCLP dichroic mirror). Emitted light was measured at 480 nm, the peak emission wavelength for ECFP, and at 535 nm (D480/30 and D535/25 emission filters), the peak emission wavelength for ECFP. Background fluorescence was determined for each plate of cells by taking readings in a region of the plate that contained no cells, using the same size field of view. These readings were subtracted from each data point. We did not subtract the autofluorescence from untransfected cells because cell densities differed between dishes. Data were expressed as either the raw fluorescence intensity or as the ratio of fluorescence intensity at 535 nm/480 nm.

**RESULTS**

**Expression of Fluorescent Fusion Proteins in Transfected CHO Cells**—Western blot analysis was used to verify that transfected CHO cells contained the appropriate fusion proteins. The proteins from the cell lysate were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-GFP antibody. Lanes were loaded from left to right with native CHO cell lysate, CHO cells expressing RII-EYFP, CHO cells expressing Ht31-ECFP, or CHO cells expressing Ht31P-ECFP. Molecular mass markers are on the left. The solid arrow points to RII-EYFP, and the dashed arrow points to Ht31-ECFP. Bands below these arrows are degradation products.
collected from five plates of cells that were stably transfected with either Ht31-ECFP or Ht31P-ECFP and then transiently transfected with RII-EYFP to determine whether cells stably expressing Ht31-ECFP or Ht31P-ECFP transiently expressed equal levels of RII-EYFP. Proteins from the cell lysate were separated by SDS-polyacryl-}



**Fig. 2.** Subcellular distribution of RII-EYFP and Ht31-ECFP when expressed in CHO cells. Cells expressing RII-EYFP (A) or Ht31-ECFP (B) were plated, prepared, and viewed as described under “Experimental Procedures.”

was a dramatic increase in fluorescence emitted at 535 nm in cells expressing Ht31-ECFP and RII-EYFP, because the intensity of the 535-nm fluorescence from these cells was 5 times greater than native CHO cells and 2 times greater than control cells expressing Ht31P-ECFP and RII-EYFP (Fig. 4). Emission at 480 nm was reduced by 34% in Ht31-ECFP/RII-EYFP expressing cells when compared with control CHO cells expressing Ht31P-ECFP and RII-EYFP. Cells expressing RII-EYFP alone displayed background levels of fluorescence when excited at 440 nm, because emission at 480 or 535 nm was reduced by an average of 97% or 84%, respectively, when compared with cells expressing both Ht31-ECFP and RII-EYFP (data not shown). Interestingly, when cells expressing Ht31-ECFP were excited at 440 nm, emission at 535 nm was only 18% lower than emission from cells expressing Ht31P-ECFP and RII-EYFP (data not shown).

To control for different densities of cells on each plate, the ratio of fluorescence at 535 nm/480 nm was used to compare FRET between cells expressing RII-EYFP and Ht31P-ECFP or RII-EYFP and Ht31P-ECFP. The ratio of fluorescence was over 2 times greater in cells expressing Ht31P-ECFP and RII-EYFP compared with cells transfected with Ht31P-ECFP and RII-EYFP (n = 18) (Fig. 5). These results are consistent with the findings of Miyawaki et al. (16) who report an ~1.5-fold ratio increase with the ECFP/EYFP pair when their “yellow cameleon” (ECFP and EYFP linked together with calmodulin
and the calmodulin-binding peptide M13) binds Ca\(^{2+}\) in response to addition of ionomycin.

**DISCUSSION**

Our FRET measurements in CHO cells co-transfected with Ht31-ECFP and RII-EYFP demonstrate a significant increase in energy transfer (i.e., an increase in the 535/480-nm emission ratio) as compared with cells transfected with Ht31P-ECFP and RII-EYFP. The efficiency (\(E\)) of FRET decreases with the sixth power of the distance (\(R\)) between donor and acceptor according to the relationship, \(E = \left(1 + \frac{R}{R_0}^6\right)^{-1}\) (18). \(R_0\), the critical Forster radius, is the distance at which FRET is 50% efficient (20). For the ECFP-EYFP pair, \(R_0\) is reported to be 50 Å (19). Thus, significant energy transfer should only occur between Ht31-ECFP and RII-EYFP if these two species are ~10–50 Å apart. We observed energy transfer between Ht31-ECFP and RII-EYFP; therefore, we can conclude that the Ht31-ECFP and RII-EYFP expressed in the CHO cells are sufficiently close for interaction between these molecules to take place.

N-terminal dimerization of RII is required for RII docking to AKAPs (21, 22) and for RII binding to Ht31 (23). Therefore, our results imply that RII-EYFP associates to form dimers in the CHO cells. Because Ht31 can bind to the N termini of dimerized RII in vitro (23, 24), the ECFP tag at the C terminus of RII should not interfere with AKAP (or Ht31) binding to RII. We tested this hypothesis using surface plasmon resonance. We assessed whether significant changes in \(K_d\) occurred as a result of adding a GFP derivative to the C terminus of these molecules. Our results showed that the affinity of RII for Ht31 was not significantly altered by fusion of EYFP to the C terminus of RII. Likewise, taking into consideration that these experiments were performed using two different SA surfaces, the \(K_d\) for Ht31-ECFP binding to immobilized RII was similar to that of RII binding to immobilized Ht31. Thus, our results demonstrate that not only can we successfully use FRET to measure the interaction between molecules of RII and the RII-binding domain of AKAPs in intact cells but also that the addition of the 27-kDa ECFP or EYFP fluorescent tag to the C terminus of the expressed proteins does not significantly alter this intercellular interaction. Thus, we can conclude that the C-terminal tag does not affect the interaction between Ht31 and RII.

In previous FRET studies, two different pairs of GFP derivatives were used (25–27). We used the ECFP-EYFP pair rather than enhanced blue fluorescent protein with enhanced GFP. One disadvantage of the ECFP-EYFP pair is that the amplitude of the change in the emission ratio is reduced, compared with the enhanced blue fluorescent protein-enhanced green fluorescent protein pair, because the ECFP emission is relatively broad and overlaps the EYFP emission spectrum (27). However, the ECFP-EYFP pair has improved photostability (16, 19). In our study, overlap of ECFP emission into the EYFP channel, referred to as “cross-talk” by Gordon et al. (28), may account for elevated FRET measurements in cells expressing the negative control Ht31P-ECFP and RII-EYFP or in cells expressing Ht31-ECFP alone. Also, autofluorescence, believed to be primarily from flavoproteins with peak excitation at 488 nm and peak emission at ~520 nm (15), may have contributed to background fluorescence in both the RII-EYFP/Ht31-ECFP and the RII-EYFP/Ht31P-ECFP-expressing cells. Thus, there is likely to be a contribution both from FRET and from autofluorescence to the 535/480-nm emission ratio for both sets of transfected cells.

In summary, the results of this study provide the first direct demonstration of binding of RII to the RII-binding domain of an AKAP in intact cells, thereby demonstrating the physiological relevance of previous measurements of RII/Ht31 interactions performed in vitro (12). Our findings indicate the potential of FRET, between expressed GFP fusion proteins, as a powerful tool for measuring PKA/AKAP interactions in the intracellular environment.

Acknowledgments—We acknowledge Dr. Jianjie Ma for helpful discussions and Russ Desnoyer for help in creating the fluorescent protein
fusion constructs. We would also like to thank Dr. Dianne Perez for critical review of the manuscript. The Becton-Dickinson FACSVantage used to perform sorting of EYFP-expressing cells was purchased through a generous gift from the Keck Foundation. ECFP-expressing cells were sorted using an Elite ESP at the Cancer Research Center at Case Western Reserve University. Polymerase chain reaction products were sequenced, and peptides were made by the Molecular Biotechnology Core facility at the Cleveland Clinic Foundation.

REFERENCES

1. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080
2. Dell’Acqua, M. L., and Scott, J. D. (1997) J. Biol. Chem. 272, 12881–12884
3. Tsunoda, S., SierraLta, J., and Zuker, C. S. (1998) Curr. Opin. Genet. Dev. 8, 419–422
4. Rubin, C. S. (1994) Biochim. Biophys. Acta 1224, 467–479
5. Ndubuka, C., Li, Y., and Rubin, C. S. (1993) J. Biol. Chem. 268, 7621–7624
6. Chen, Q., Lin, R. Y., and Rubin, C. S. (1997) J. Biol. Chem. 272, 15247–15257
7. Yang, J., Drazba, J., Ferguson, D., and Bond, M. (1998) J. Cell Biol. 142, 511–522
8. Lester, L. B., Langeberg, L. K., and Scott, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14942–14947
9. Gao, T., Yatani, A., Dell’Acqua, M. L., Sako, H., Green, S. A., Dascal, N., Scott, J. D., and Hasey, M. M. (1997) Neuron 19, 185–196
10. Lohmann, S. M., DeCamilli, P., Eving, I., and Walter, U. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6723–6727
11. Carr, D. W., Skofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, B. G., and Scott, J. D. (1993) J. Biol. Chem. 268, 14188–14192
12. Carr, D. W., Hausken, Z. E., Fraser, I. D. C., Skofko-Hahn, R. E., and Scott, J. D. (1992) J. Biol. Chem. 267, 13376–13382
13. Hausken, Z. E., Coghlan, V. M., Hastings, C. A., Reimann, E. M., and Scott, J. D. (1994) J. Biol. Chem. 269, 24245–24251
14. Newlon, M. G., Roy, M., Morikis, D., Hausken, Z. E., Coghlan, V., Scott, J. D., and Jennings, P. A. (1999) Nat. Struct. Biol. 6, 222–227
15. Benson, R. C., Meyer, R. A., Zaruba, M. E., and McKhann, G. M. (1979) J. Histochem. Cytochem. 27, 44–48
16. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Nature 388, 882–887
17. Hausken, Z. E., and Scott, J. D. (1996) Biochem. Soc. Trans. 24, 986–991
18. Zakhary, D. R., Moravec, C. S., Stewart, R. W., and Bond, M. (1999) Circulation 99, 505–510
19. Heim, R., and Tsien, R. Y. (1996) Curr. Biol. 6, 178–182
20. Wu, P., and Brand, L. (1994) Anal. Biochem. 218, 1–13
21. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005
22. Banyk, P., Huang, L. S., and Taylor, S. S. (1998) J. Biol. Chem. 273, 35048–35055
23. Newlon, M. G., Roy, M., Hausken, Z. E., Scott, J. D., and Jennings, P. A. (1997) J. Biol. Chem. 272, 23657–23664
24. Zhao, J., Hoye, E., Boylan, S., Walsh, D. A., and Trewhella, J. (1998) J. Biol. Chem. 273, 30448–30459
25. Romoser, V. A., Hinkle, P. M., and Persechini, A. (1997) J. Biol. Chem. 272, 13270–13274
26. Mahajan, N. P., Linke, K., Berry, G., Gordon, G. W., Heim, R., and Herman, B. (1998) Nat. Biotechnol. 16, 547–552
27. Pollock, B. A., and Heim, R. (1999) Trends Cell Biol. 9, 57–60
28. Gordon, G. W., Berry, G., Liang, X. H., Levine, B., and Herman, B. (1998) Biophys. J. 74, 2702–2713