Oligomerization of Transcriptional Intermediary Factor 1
Regulators and Interaction with ZNF74 Nuclear Matrix
Protein Revealed by Bioluminescence Resonance Energy
Transfer in Living Cells*

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Transcriptional intermediary factor 1 (TIF1) α and
KAP-1/TIF1β, two members of the TIF1 family of nuclear
cofactors, are ubiquitous co-regulators of nuclear recep-
tors and KRAB motif-containing zinc finger transcription
factors, respectively. Despite the functional evidence
suggesting a role for TIF1 proteins as modulators of
transcription, the study of their interactions with
transcriptional machineries in physiologically relevant
systems has been difficult. Here, we have developed a
bioluminescence resonance energy transfer (BRET) bio-
physical approach to study protein-protein interactions
in the nuclear compartment of living mammalian cells.
We report that TIF1α and KAP-1 form homo- and hetero-
oligomers in intact mammalian cells. BRET titration ex-
eriments indicate that both homo- and hetero-
oligomers have relatively high affinity suggesting
that could co-exist in cells. Furthermore, we dem-
strate that KAP-1 but not TIF1α interacts with the
KRAB multifinger ZNF74 in the nuclear matrix. Splice
variants and point mutants of ZNF74 that lack tran-
scriptional activity were found not to interact with
KAP-1 confirming the physiological importance of this
interaction in living cells. The interaction of ZNF74 with
KAP-1 did not prevent KAP-1 homomerization indicat-
ing that the oligomers most likely represent the tran-
scriptionally active species. Furthermore, the detection
of ternary ZNF74/KAP-1/TIF1α complexes suggests the
existence of cross-talk between KAP-1-interacting
KRAB proteins and TIF1α-interacting nuclear recep-
tors. In addition to providing new insights into the
molecular interactions involved in the transcriptional
activities of these proteins, this study shows that BRET
can be advantageously used as a non-transcription-
based oligomerization detection system to study the
interaction of transcriptionally active proteins, including
nuclear matrix proteins, in living cells.

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TIF1α and KAP-1 (TIF1β) are ubiquitously expressed mem-
bers of the transcriptional intermediary factor 1 (TIF1) family.
TIF1α was described as a modulator of ligand-activated tran-
scription mediated by the retinoid nuclear receptors RXR
and RAR (1–5). KAP-1, for its part, has been proposed to act as a
co-repressor for several KRAB motif-containing zinc finger
transcription factors (6) such as human KOX1 (7, 8), ZNF133
and ZNF140 (7), rat Kid1 (9), and mouse KRAZ1 and KRAZ2
(10). Although largely distributed in vertebrates, the absence
of KRAB motif-containing proteins and TIF1 family members in
yeast Saccharomyces cerevisiae suggests a late evolutionary
appearance and expansion of these two protein families (8, 11).

Whereas a few studies have suggested that TIF1 family
members can act as homo- or hetero-oligomers (12), the nature
of the complexes in which these proteins are engaged remain
largely unknown. Indeed, despite the evidence suggesting an
important role for TIF1 proteins as modulator of transcription,
the study of their interactions with the transcriptional machin-
ery in physiologically relevant systems has been difficult. Stud-
ies addressing their physical interactions included in vitro
assays carried out mainly with soluble fragments (to minimize
aggregation) of recombinant proteins (1, 3, 4, 12–16), transcrip-
tion-based yeast two-hybrid assays (1, 3, 5, 8, 9, 17) and co-
immunoprecipitation in overexpression systems (4, 14, 18).
However, results obtained with these various approaches led
in conflicting conclusions. For instance, in vitro and co-immu-
oprecipitation studies suggested that TIF1α and KAP-1 can
homo-oligomerize (14) although no such homophilic interaction
was detectable in yeast two-hybrid interaction assays (8). Con-
versely, whereas heteromerization of TIF1α with KAP-1 was
evidenced by yeast two-hybrid methods (5), it was not detected
using in vitro and co-immunoprecipitation approaches (14).

The analysis of the interactions involving TIF1 family mem-
bers is further complicated when considering that the tran-
scriptional activities of TIF1 and KRAB-containing proteins
dramatically differ between yeast, where these proteins are not
normally expressed, and mammalian cells. In contrast to what
is found in mammalian cells, TIF1α, KAP-1, nor KRAB do-
main-containing proteins have transcriptional repressor activity
in yeast (8) with the consequence that many functionally
relevant interactions may not be found in this system (2).

In one case, a physiologically more appropriate transcrip-
Transcriptional Regulator Oligomers Revealed in Vivo by BRET

Human embryonic kidney 293T cells (32) maintained in Dulbecco’s modified Eagle’s medium supplemented by 10% fetal bovine serum (Invitrogen), 100 μg/ml penicillin and streptomycin, 1 mM t-glutamine were seeded at a density of 1 × 10^6 cells per 100-mm dish. Transient transfections of plasmids were performed the following day by using the calcium phosphate precipitation method (30).

BRET Assay

Transient transfections with RLuc and/or GFP10 fusion or other constructs were performed in 293T as described above. The total amount of DNA used for transfection was adjusted to 10 μg by adding pGEM4 vector (Promega). Forty-two hours post-transfection (or 48 h for transfections including TIF1α constructs), cells were detached with 2 mM phosphate-buffered saline/EDTA and washed twice with 1 mM phosphate-buffered saline/glucose. Cells were resuspended in 1 mM phosphate-buffered saline/glucose at ~2 × 10^6 cells/ml and 40 μl (20 μg) were distributed in 96-well microplates (white Optiplate from Packard). Upon the addition of 5 μM of the cell permeant luciferase substrate, coelenterazine deep blue (10 μl) (PerkinElmer Life Sciences), the bioluminescence resulting from its degradation (emission peak 400 nm) was detected using a 370–450-nm band pass filter (donor emission). The energy transferred to GFP10 (emission peak 510 nm) was detected using a 500–530-nm band pass filter. Readings were collected with a modified total count apparatus (BRETCount, PerkinElmer Life Sciences) that allows sequential integration of the signals detected at 370–450 nm (bioluminescence signal) and at 500–530 nm (fluorescence signal). The BRET signal (BRET ratio) was quantified by calculating the fluorescence/luminescence ratio as previously reported (33). The BRET ratio was found to be stable over several readings performed at different times after addition of the substrate (here evaluated in a 5–20-min range) (23). Expression level of each construct was determined by direct measurements of total fluorescence and luminescence on aliquots of transfected cell samples. The GFP10 total fluorescence was measured using a FluoroCount (PerkinElmer Life Sciences) with an excitation filter at 400 nm, an emission filter at 510 nm, and the following parameters: gain 1; PMT 1100 V; time 1.0 s. After the fluorescence measurement, the same cells were incubated for 10 min with coelenterazine H (Molecular Probes) at a final concentration of 5 μM and the total luminescence of cells was measured using a Lumicount (Biosystems, Flow Cytometry Life Sciences) with the following parameters: gain 1; PMT 700 V; time 0.5 s. In contrast to deep blue coelenterazine, coelenterazine H does not lead to energy transfer to GFP10 and thus allows assessment of RLuc activity without GFP10 emission quenching. The BRET ratios were plotted as a function of the GFP/FLUC fusion protein expression ratio, both fusion proteins expression being assessed with the same cells as described above, to take into account the potential variations in the expression of individual constructs from transfection to transfection. BRET titration curves were fitted using a non-linear regression equation (GraphPad Prism).

Expression Vectors

KAP-1 and TIF1α Constructs—Full-length KAP-1 (accession number NM_011588; aa 1–835) was derived from pMPF5-Gal4-KAP1 construct (generous gift from Dr. J. V. Bonventre) (9, 25) and subcloned as an EcoRI-Nol fragment into the EcoRI and EcoRV sites of cytomegalovirus-driven pHRLuc C3 and pGFP10 C2 vectors, respectively (PerkinElmer Life Sciences). Full-length TIF1α (accession number S78219; aa 1–1017) was isolated from pSG5-TIF1α construct DNAs coding for ZNF74-I (accession number NM_011588; aa 1–572) (27), ZNF74-II (accession number NM_011588; aa 1–643) (28), and the zinc finger domain of ZNF74 (ZN) (aa 175–289) was isolated from pSG5-Gal4-KAP1 construct (generous gift from Dr. R. Losson) (4) and subcloned as a BamHI fragment into pHRLuc C2 and pGFP10 C1 BamHI sites. For in-phase cloning after an NH2-terminal HA tag, full-length KAP-1 and TIF1α were cloned into the blunted BamHI or XbaI sites of cytomegalovirus-driven pCGN vector (26), respectively.

ZNF74 Constructs—DNAs for ZNF74-I (accession number AF176233; aa 1–572) (27), ZNF74-II (accession number NM_011588; aa 1–643) (28), and the zinc finger domain of ZNF74 (ZN) (aa 175–509 when numbered relative to ZNF74-I or aa 246–580 when numbered relative to ZNF74-II) were derived from clones previously obtained and cloned in pCGN (27, 28). To construct the KRAB mutant of ZNF74-II (D474A/V484A), subcloning of the KRAB domain-conserved DV amino acids (29) to alanine residues was obtained by PCR-mediated mutagenesis (30) and the KRAB-containing region of ZNF74-II was replaced by the corresponding fragment containing the mutation. For BRET constructs, all ZNF74 sequences were subcloned as XbaI fragments into the pHR-Luc C1 and pGFP10 C3 XbaI sites at the COOH terminus of luciferase and GFP (pHRLuc C1 ΔXhol/BamHI blunted, pGFP10 C3 ΔSanU/ BamHI blunted). pMal-c vector (New England Biolabs) was used to express maltose-binding protein (MBP) in fusion with ZNF74 proteins as previously described (27).

BRET fluorescence and bioluminescence resonance energy transfer (FRET or BRET) have recently been used to assess protein–protein interactions in living cells (20, 21). In particular, BRET was successfully used to study homomerization of the Kai transcription factors, in the nuclear-less bacterial cell Escherichia coli (22), and the oligomerization of the membrane-bound G protein-coupled membrane receptors in mammalian cells (23, 24). Here, using nuclear and nuclear-matrix targeting sequences as controls, BRET was used for the first time to quantitatively study protein interactions in the nuclear compartments of living mammalian cells.

We report that two TIF1 family members, TIF1α and KAP-1, can form homo- and hetero-oligomers in intact mammalian cells and that KAP-1, unlike TIF1α, interacts with the KRAB multifinger protein ZNF74 in the nuclear matrix. In addition to providing new insights into the molecular interactions involved in the transcriptional activities of the TIF1 and KRAB-containing proteins, our study demonstrates that BRET can advantageously be used as a non-transcription-based detection system to study the interactions of full-length transcriptional regulators, including nuclear matrix proteins, in living cells.
by the washed affinity resin were analyzed by SDS-polyacrylamide gel electrophoresis and electrotitrated for Western blotting. After the anti-HA immunodetection, the blot was dehybridized and rehybridized with an anti-MBP antibody to confirm the loading of equivalent amounts of MBP fusion proteins (data not shown).

Western Blotting

The expression of epitope-tagged and fusion proteins immobilized on nitrocellulose membranes was verified by Western blotting using the 12CA5 mouse anti-HA antibody (35), rabbit polyclonal anti-GFP (Clontech), rabbit anti-MBP (New England Biolabs), rabbit anti-ZNF74 antibody raised against aa 381 to 564 of ZNF74-II, and mouse polyclonal anti-KAP1 raised against aa 381 to 564 (36). Following addition of the appropriate secondary antibody (either a sheep anti-mouse or goat anti-rabbit horseradish peroxidase), a chemiluminescence reagent was used (Renaissance kit, Amersham Biosciences).

RESULTS AND DISCUSSION

Oligomerization of TIF1 Family Members Detected in Living Cells—Although the presence of interaction motifs such as a ring finger, B boxes, and a coiled-coil domain (RBCC) suggests that members of the TIF1 family could oligomerize, studies assessing such interactions led to conflicting results (5, 8, 12–14). Here, using a newly developed BRET assay (22, 23, 33), homo- and heteromerization of KAP-1 and TIF1α were investigated in living mammalian cells. Because the transfer of energy between a bioluminescence donor luciferase and a fluorescent acceptor GFP occurs with a R0 of ~50 Å and that no transfer would be detected for distances above 100 Å (22), BRET was used to monitor intermolecular interactions between these potential partners. To this end, full-length KAP-1 and TIF1α were tagged at their amino terminus with either Renilla reniformis luciferase (Rluc) or Aequorea GFP green variant (GFP10) (Fig. 1A). Homomeric and heteromeric pairs were then co-expressed in 293T cells and the occurrence of BRET was determined by measuring the ratio of the light emitted by the GFP (410±40 nm) and the luciferase (515±15 nm) upon addition of the membrane-permeable luciferase substrate coelenterazine. The BRET ratio was plotted as a function of the GFP/Luc fusion protein expression ratio (both fusion proteins expressed from transfection to transfection. As shown in Fig. 1A, significant BRET ratios, indicative of protein proximity, were detected for the Rluc-KAP1/GFP10-KAP1, Rluc-TIF1αluc-GFP10-TIF1α, and Rluc-TIF1αluc-GFP10-KAP1 pairs. In each case, when a fixed amount of Rluc fusion was transfected, the BRET ratio increased as a function of the amount GFP fusion transfected and reached a maximum when the amount of expressed GFP fusion (acceptor) was no longer limiting compared with Rluc fusion (donor). Such saturation is indicative of a specific interaction. When considering the heteromeric TIF1αluc/KAP1 pair, identical results were obtained for the two possible BRET orientations (i.e. Rluc-TIF1αluc/GFP10-KAP1 and Rluc-KAP1/GFP10-TIF1αluc; data not shown). Because as most techniques, BRET cannot distinguish between dimers and higher order oligomeric species, the term oligomer is used to describe the detected interactions with the understanding that some of the complexes may be simple dimers.

To ascertain the specificity of the KAP-1 and TIF1α oligomers detected, BRET was measured in cells co-expressing Rluc-KAP1 or Rluc-TIF1αluc and a construct targeting cytoplasmic GFP to the nucleus by fusion to a nuclear localization signal (GFP10-NLS) construct. The efficient nuclear targeting of GFP10-NLS, GFP10-TIF1αluc, and GFP10-KAP1 was confirmed by immunofluorescence microscopy (not shown). Only marginal BRET, most likely resulting from random collision (“bystander” BRET (33)), was obtained with the Rluc-KAP1/GFP10-NLS pairs. 293T cells were transfected with Rluc-KAP1 (1 μg) and variable amounts of GFP10-KAP1 (0.05 to 3 μg) (●) or negative control GFP10-NLS (0.1 to 3 μg) (■). Transfections were also performed with a fixed amount of Rluc-TIF1αluc (2 μg) and variable amounts of GFP10-TIF1αluc (0.1 to 25 μg) (○) or GFP10-KAP1 (0.1 to 3 μg) (□). The BRET ratio determined after adding coelenterazine (blue is represented as a function of the GFP over luciferase fusion expression (GFP10/Luc), the luciferase donor and GFP10 acceptor expression levels being determined as described under “Experimental Procedures.” The BRET0.5 and BRETmax are 0.40±0.15 and 0.20±0.02 for the KAP-1 homophilic fusion pair, 0.32±0.07 and 0.24±0.01 for the TIF1αluc homophilic pair, and 0.74±0.31 and 0.16±0.03 for the TIF1αluc/KAP1 pair, respectively. For each pair, data of a minimum of three independent experiments were pooled. B, specificity of the BRET signal obtained with KAP-1 and TIF1αluc. 293T cells were transfected either with Rluc-KAP1 (1 μg) and GFP10-KAP1 (2 μg) or Rluc-TIF1αluc (1 μg) and GFP10-TIF1αluc (5 μg) to obtain a BRET ratio close to the maximal as determined in A. Competing HA-tagged-KAP1 expression vectors were also transfected as indicated; the expression of the HA-tagged protein was assessed by immunoblot (IB) as represented above the bar graph. No significant change in the relative expression level of Rluc and GFP fusions that could account for change in the BRET ratio was observed in the presence of competitor as evaluated by the GFP10/Luc ratio indicated. The data shown are representative of three independent experiments. C, KAP-1 oligomerization detected by co-immunoprecipitation. 293T cells were transfected with GFP10-KAP1 (2.5 μg) and/or HA-KAP1 (2.5 μg). Cell lysates and HA antibody-immunoprecipitated proteins (IP) were analyzed by immunoblotting with anti-HA- and anti-GFP-specific antibodies. The fractions of the cell lysate (1/100) and the immunoprecipitated proteins (1/10 or 9/10) used are indicated.

FIG. 1. Oligomerization of two members of the TIF1 family, KAP-1 and TIF1α. A, KAP-1 and TIF1α oligomerization assessed by BRET titration in living mammalian cells. Full-length KAP1 and TIF1α were used as fusion proteins with Rluc and GFP10 as schematized. 293T cells were transfected with a fixed amount of Rluc-KAP1 (1 μg) and variable amounts of GFP10-KAP1 (0.05 to 3 μg) (●) or negative control GFP10-NLS (0.1 to 3 μg) (■). Transfections were also performed with a fixed amount of Rluc-TIF1αluc (2 μg) and variable amounts of GFP10-TIF1αluc (0.1 to 25 μg) (○) or GFP10-KAP1 (0.1 to 3 μg) (□). The BRET ratio determined after adding coelenterazine (blue is represented as a function of the GFP over luciferase fusion expression (GFP10/Luc), the luciferase donor and GFP10 acceptor expression levels being determined as described under “Experimental Procedures.” The BRET0.5 and BRETmax are 0.40±0.15 and 0.20±0.02 for the KAP-1 homophilic fusion pair, 0.32±0.07 and 0.24±0.01 for the TIF1αluc homophilic pair, and 0.74±0.31 and 0.16±0.03 for the TIF1αluc/KAP1 pair, respectively. For each pair, data of a minimum of three independent experiments were pooled. B, specificity of the BRET signal obtained with KAP-1 and TIF1αluc. 293T cells were transfected either with Rluc-KAP1 (1 μg) and GFP10-KAP1 (2 μg) or Rluc-TIF1αluc (1 μg) and GFP10-TIF1αluc (5 μg) to obtain a BRET ratio close to the maximal as determined in A. Competing HA-tagged-KAP1 expression vectors were also transfected as indicated; the expression of the HA-tagged protein was assessed by immunoblot (IB) as represented above the bar graph. No significant change in the relative expression level of Rluc and GFP fusions that could account for change in the BRET ratio was observed in the presence of competitor as evaluated by the GFP10/Luc ratio indicated. The data shown are representative of three independent experiments. C, KAP-1 oligomerization detected by co-immunoprecipitation. 293T cells were transfected with GFP10-KAP1 (2.5 μg) and/or HA-KAP1 (2.5 μg). Cell lysates and HA antibody-immunoprecipitated proteins (IP) were analyzed by immunoblotting with anti-HA- and anti-GFP-specific antibodies. The fractions of the cell lysate (1/100) and the immunoprecipitated proteins (1/10 or 9/10) used are indicated.
were measured to control that the change in BRET ratios did not result in toxic levels for the cells. Indeed, as shown in Fig. 1A, at least 15% of the total GFP-KAP1 could be co-immunoprecipitated with HA-KAP1. The low level of TIF1 

eluc-KAP1/GFP10-KAP1, BRET$_{50} = 0.40 \pm 0.15$) tend to have a slightly higher binding affinity than the heteromeric pair (Rluc-TIF1a/GFP10-KAP1, BRET$_{50} = 0.74 \pm 0.31$). In an attempt to determine the molar ratio of GFP10/Rluc constructs needed to reach the BRET$_{50}$, a construct covalently fusing the Rluc and the GFP10 (Rluc-GFP) was used. The GFP/LUC expression ratio obtained for this fusion, which by definition corresponds to an equimolar concentration of GFP10 and Rluc, was 0.125 $\pm$ 0.020 arbitrary units ($n = 5$). Assuming that this value can be used to determine the ratio of the various Rluc and GFP10 fusions tested, 2.5, 3, and 6 times more GFP fusion than Rluc fusion was required to reach half the maximum BRET ratio for TIF1a homomeric, KAP-1 homomeric, and TIF1a/KAP-1 heteromeric pairs, respectively. These data indicate that the affinities between the partners are relatively high and that large excess is not needed for the interactions to occur. Furthermore, because the affinity of the homo- and heteromers are in the same range of magnitude, these complexes can potentially co-exist in living cells.

Co-immunoprecipitation experiments carried in 293T cells co-expressing the differentially tagged full-length KAP-1 proteins, GFP10-KAP1 and HA-KAP1, confirmed that KAP-1 oligomers represent stable complexes that resist cell lysis and sample preparation. Indeed, as shown in Fig. 1C, at least 15% of the total GFP-KAP1 could be co-immunoprecipitated with the HA-KAP1. The low level of TIF1a expression did not allow co-immunoprecipitation experiments to be carried out using up to 1 mg of cell extract. Starting with larger amounts of cell extracts (~4 mg of protein), Peng et al. (14) were able to detect TIF1a homomerization by co-immunoprecipitation but failed to detect interaction between TIF1a and KAP-1 (14). The detection by BRET of both TIF1a homo- and heteromerization with KAP-1, using as little as 100,000 cells (~20 $\mu$g of protein) illustrates the high sensitivity of the energy transfer assay.

![Fig. 2. Interactions between ZNF74, KAP-1, and TIF1a in living mammalian cells.](http://www.jbc.org/)

**A** BRET titration curves. Full-length KAP-1, TIF1a, and ZNF74-II (an isoform of ZNF74 with a functional repressive KRAB box) were used. 293T cells were transfected with a fixed amount of Rluc-ZNF74-II (1 $\mu$g) and variable amounts of GFP10-KAP1 (0.05 to 2 $\mu$g), GFP10-TIF1a (0.5 to 15 $\mu$g), or negative control GFP10-NLS (0.05 to 1 $\mu$g) (C, dotted line). The BRET ratio is represented as a function of the GFP over luciferase fusion expression. For the ZNF74-II/KAP-1 pair, the BRET$_{50}$ and BRET$_{max}$ are 0.48 $\pm$ 0.12 and 0.35 $\pm$ 0.03, respectively. For each curve, results of at least three independent experiments were pooled. **B** specificity of the interaction of ZNF74-II with KAP-1. 293T cells were transfected with the Rluc-ZNF74-II (1 $\mu$g)/GFP10-KAP1 (2 $\mu$g) pair or Rluc-KAP1 (1 $\mu$g)/GFP10-KAP1 (2 $\mu$g) pair in the presence or absence of competitor. For competition, expression vectors for either HA-KAP1 or HA-ZNF74-II were used at the indicated amounts. In addition to the BRET ratios, GFP/LUC ratios were measured to control that the change in BRET ratios did not result from alterations in the relative expression level of Rluc and GFP fusions. Immunoblot analysis further confirmed the equivalent expression levels of fusion proteins. For immunoblots (IB), HA-KAP or HA-ZNF74 competitor expression was detected with anti-HA, GFP10 fusion with anti-GFP, and Rluc fusions either with anti-ZNF74 or anti-KAP1.

**C** BRET signal induction by formation of ternary complexes involving ZNF74-II/HAP-1/KAP-1. 293T cells were transfected with Rluc-TIF1a (2 $\mu$g) and GFP10-ZNF74-II (5 $\mu$g) in the presence or absence of HA-KAP1. Immunoblots for HA-tagged KAP1 and GFP/LUC ratios are shown. For B and C, the data shown are representative of three independent experiments.
Transcriptional Regulator Oligomers Revealed in Vivo by BRET

Considering the conflicting results obtained relative to the homo- and heteromerization of TIF1 family members using yeast two-hybrids, in vitro studies, and co-immunoprecipitation (as pointed out in the introduction), BRET provides here a unique means to assess these interactions and unambiguously demonstrates that TIF1α and KAP-1 form homo- and heteromers in intact mammalian cells.

The functional significance of TIF1 family member oligomerization still remains to be determined. However, it was found that purified fragments of the KAP-1 RBCC domain can oligomerize and that such oligomerization is required for interaction with the purified KRAB repressor domain of Kox-1 in vitro (13). Assuming that native proteins also depend on KAP-1 oligomerization for interaction, this suggests that the KAP-1 co-repressor oligomerization may be a sine qua non condition for KRAB proteins to exert their repressive function. From a study showing that TIF1α coimmunoprecipitates with TIF1α and prevents TIF1α repression of RXR nuclear receptor-mediated transcription, it was also suggested that the heteromerization of some TIF1 family members may constitute a transcription regulatory mechanism (14). Also, it was recently suggested that proper nuclear targeting of KAP-1 to transcriptionally silent centromeric regions may require its oligomerization (18). Indeed, this targeting was compromised by altering the RBCC domain of KAP-1 but preserved by replacing the RBCC domain with GAL4 DNA binding domain, which has the ability to dimerize. Whether the TIF1α/KAP1 heteromerization shown in this study affects intranuclear localization of these individual TIF1 family members, or their respective transcriptional activity, remains to be determined.

Interactions between ZNF74, KAP-1, and TIF1α in Living Cells—ZNF74 is a nuclear matrix protein (27) that belongs to the large KRAB domain-containing multigene family. Two isoforms, a long one (ZNF74-II) containing a full KRAB box and a shorter one (ZNF74-I) with an incomplete KRAB domain, are generated by alternative promoter usage and splicing (28). Whereas ZNF74-I is preferentially located to nuclear speckles enriched in splicing factors and is transcriptionally inactive, ZNF74-II has a more diffuse nuclear localization and its KRAB box has been shown to repress transcription (28). Given that the repressor activity of several KRAB multigene proteins has been shown to require an interaction with the co-repressor KAP-1 (7), one could propose that ZNF74-II also mediates repression through a direct interaction with KAP-1. However, because of its tight attachment to the nuclear matrix and its intrinsic repressive activity, the in vivo interactions of ZNF74 with KAP-1 (or other candidate protein partners) could not be readily assessed in mammalian cells either by co-immunoprecipitation or by a transcription-based interaction assay. Here thus, BRET was used to assess the interaction between ZNF74-II and KAP-1 in living mammalian cells. As seen in Fig. 2A, significant BRET ratios revealing heteromerization of ZNF74-II and KAP-1 were obtained for the RuLuc-ZNF74-II/GFP10-KAP1 pair. Similar BRET ratios were obtained when the partners were tested in the reverse orientation (RuLuc-KAP1/GFP10-ZNF74-II; see Fig. 3A). In contrast, no heteromerization between ZNF74-II and the related TIF1α was detected. Indeed, the marginal BRET signals obtained between RuLuc-ZNF74-II and GFP10-TIF1α (or the reverse pair; not shown) was not different from the background signal observed with the negative control RuLuc-ZNF74-II/GFP10-NLS pair (Fig. 2A).

The specificity of RuLuc-ZNF74-II/GFP10-KAP1 heteromerization was confirmed by the reduction in the BRET ratio observed upon increasing concentrations of competing HA-KAP1 (Fig. 2B). Interestingly, no competition occurred when
HA-ZNF74-II was added to the Rluc-KAP1/GFP10-KAP1 pair and thus, the formation of BRET productive homomers of KAP-1 is not impaired by the interaction of ZNF74-II with KAP-1. This indicates that ZNF74-II can bind to oligomers of KAP-1 in intact mammalian cells and suggests that the oligomers most likely represent the transcriptionally active species. Such interaction between ZNF74 and oligomers of KAP-1 is consistent with the previous in vitro finding that purified RBCC fragments from KAP-1 interacted as multimers with a KRAB fragment from KOX1 (12, 13).

The interaction of one KRAB zinc finger protein, KOX1, with TIF1α (5, 8, 17) previously led to the suggestion that cross-talk could exist between KRAB multimeric proteins and nuclear receptors known to interact with TIF1α (11, 37). However, our data using full-length proteins in living mammalian cells suggest that ZNF74 does not interact directly with TIF1α. Similarly, another study failed to demonstrate TIF1α interaction with the isolated KRAB domain of five different KRAB zinc finger proteins using yeast two-hybrid systems (17). Thus, for these KRAB zinc finger proteins as well as for ZNF74, a possible cross-talk with nuclear receptors is not likely to occur through a direct interaction with TIF1α. To test the hypothesis that a cross-talk regulation between TIF1α and ZNF74 could result from the formation of a ternary complex between TIF1α, ZNF74, and KAP-1, the ability of KAP-1 to promote a BRET productive interaction between TIF1α and ZNF74-II was assessed. As shown in Fig. 2C, the background BRET signal detected between TIF1α and ZNF74-II (see Fig. 2A for titration curve) was significantly potentiated by the addition of the unfused HA-KAP1. Such increase in the BRET signal was specific for the TIF1α/ZNF74 pair because it was not observed when GFP10-NLS was used instead of GFP10-ZNF74 (data not shown). This indicated that KAP-1, TIF1α, and ZNF74 are part of a protein complex placing TIF1α and ZNF74 in close enough proximity for BRET to occur. Thus, we suggest that formation of ternary complexes between the co-regulators KAP-1, TIF1α, and KRAB multimeric proteins such as ZNF74 may allow cross-talk of KRAB multimeric proteins with nuclear receptors.

**Differential Interaction of KAP1 with ZNF74 Isosforms: Requirement of an Intact KRAB Domain**—As indicated above, the BRET signal obtained between KAP1 and ZNF74 was independent of the donor/acceptor orientation considered. Indeed, as seen in Fig. 3A, the BRET saturation curve obtained with the Rluc-KAP1/GFP10-ZNF74-II pair (BRETmax = 0.39 ± 0.16; BRETmax = 0.31 ± 0.04) was indistinguishable from that obtained with the reverse pair presented in Fig. 2A (BRETmax = 0.48 ± 0.12; BRETmax = 0.35 ± 0.03). As previously shown for ZNF74 (27), the GFP10-ZNF74-II fusion was exclusively targeted to the nuclear matrix and was not detected in the soluble fraction of the nucleus including the DNAse-released chromatins (Ns) or in the cytoplasmic-enriched fraction (Cyt) (Fig. 3A). Thus, as a negative control, a minimal nuclear matrix targeting sequence (ZNF74-I(aa246–505) (38) fused to GFP was used. As expected, the fusion was exclusively recovered in the nuclear matrix and only a background signal was obtained for the Rluc-KAP1/GFP10-ZNF74-II(aa246–505) pair (Fig. 3A) ruling out that the BRET signal observed between Rluc-KAP1 and GFP10-ZNF74-II could represent bystander BRET resulting from crowding in the nuclear matrix.

Previous in vitro and yeast two-hybrid studies indicated that the KRAB domain of a few KRAB-containing proteins is necessary and sufficient for interaction with KAP-1 co-repressor (7, 9, 17). Because the ZNF74-I isoform encodes an incomplete KRAB domain deleted from its first 31 amino acids, we tested its interaction with KAP-1 in living mammalian cells. In contrast with the results obtained for ZNF74-II, the marginal BRET signal observed between Rluc-KAP1 and GFP10-ZNF74-I (Fig. 3A) (or the reverse pair, not shown) was not different from that observed with the nuclear matrix negative control. The importance of the KRAB box integrity for occurrence of the interaction was further assessed by mutating two amino acids highly conserved and proposed to be important for the interaction of KRAB zinc-finger proteins with KAP-1 (29). Mutation of aspartate 47 and valine 48 to alanine residues, within the ZNF74-II KRAB domain, abrogated the interaction with KAP-1 as indicated by the absence of significant BRET between Rluc-KAP1 and GFP10-ZNF74-II(DV→AA) (Fig. 3A). The absence of significant BRET was not because of inappropriate expression of GFP-ZNF74 constructs because similar GFP activity were observed (as accounted by the GFP/LUC ratio). The integrity of the fusion protein produced was also confirmed by immunoblot analysis that revealed the appropriate molecular weight for all constructs (Fig. 3B). Pull-down assays using bacterially expressed ZNF74 isoforms and the DV→AA mutant were in agreement with the BRET results obtained in intact cells (Fig. 3C).

The observation that the transcriptionally active ZNF74-II interacts with the corepressor KAP-1, whereas the transcriptionally silent ZNF74-I (28) does not, suggests that the repressive activity of ZNF74-II is mediated by its interaction with KAP-1. This confirms the proposed role of KAP-1 as a universal co-repressor for KRAB zinc finger proteins (19).

**CONCLUSION**

In this study, BRET is shown for the first time to represent a reliable, quantitative and highly sensitive method to assess the interaction of transcriptionally active nuclear proteins in living cells. We took advantage of this non-transcription-based method to clearly show that two members of the TIF1 family, TIF1α and KAP-1 co-regulators of transcription can homo- and heteromerize in living mammalian cells. Furthermore, we show that the ZNF74 nuclear matrix protein from the large KRAB multimeric family interacts with homomers of the corepressor KAP-1 but also with heteromers of KAP-1/TIF1α. This suggests that such heteromers may mediate cross-talk between KRAB multimeric proteins and nuclear receptors known to interact with TIF1α coregulator. As BRET allows real time kinetic studies to be performed in living cells, it will now be possible to assess such interactions under various conditions affecting cell cycle and transcriptional activity.

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