Ligand recognition and G-protein coupling selectivity of cholecystokinin A receptor
Supplementary Table 1. Cryo-EM data collection, refinement, and validation statistics.

|                              | CCK₄R/G$_q$/scFv16 (EMD-31389) | CCK₄R/G$_s$ (EMD-31388) | CCK$_i$R/G$_s$/seFv16 (EMD-31387) |
|------------------------------|--------------------------------|-------------------------|----------------------------------|
|                              | (PDB: 7EZM)                    | (PDB: 7EZK)             | (PDB: 7EZM)                      |
| Data collection and processing|                                |                         |                                  |
| Magnification                | 81,000                         | 81,000                  | 81,000                           |
| Voltage (kV)                 | 300                            | 300                     | 300                              |
| Electron exposure (e⁻/Å²)    | 80                             | 80                      | 80                               |
| Defocus range (µm)           | -0.5 ~ -3.0                    | -0.5 ~ -3.0             | -0.5 ~ -3.0                      |
| Pixel size (Å)               | 1.045                          | 1.045                   | 1.045                            |
| Symmetry imposed             | C1                             | C1                      | C1                               |
| Initial particle projections (no.) | 3,405,355                   | 4,680,972               | 4,270,010                        |
| Final particle projections (no.) | 555,628                   | 499,924                 | 140,602                          |
| Map resolution (Å)           | 2.9                            | 3.1                     | 3.2                              |
| FSC threshold                | 0.143                          | 0.143                   | 0.143                            |
| Map resolution range (Å)     | 2.3-4.3                        | 2.3-4.3                 | 2.3-4.3                          |
| Reﬁnement                   |                                |                         |                                  |
| Initial model used           | 6OIJ                           | 6NBF                    | 6OMM                             |
| (PDB accession number)       |                                |                         |                                  |
| Model resolution (Å)         | 3.0                            | 3.2                     | 3.4                              |
| FSC threshold                | 0.5                            | 0.5                     | 0.5                              |
| Map sharpening B-factor (Å²) | -97.47                         | -134.32                 | -111.38                          |
| Model composition            |                                |                         |                                  |
| Non-hydrogen atoms           | 8999                           | 7196                    | 8860                             |
| Protein residues             | 1170                           | 922                     | 1153                             |
| B-factors (Å²)               |                                |                         |                                  |
| Protein                      | 56.03                          | 66.86                   | 63.12                            |
| RMSD                         |                                |                         |                                  |
| Bond lengths (Å)             | 0.010                          | 0.010                   | 0.002                            |
| Bond angles (°)              | 1.027                          | 1.010                   | 0.625                            |
| Validation                   |                                |                         |                                  |
| MolProbity score             | 1.45                           | 1.39                    | 1.35                             |
| Clashscore                   | 4.50                           | 3.85                    | 2.45                             |
| Rotamer outliers (%)         | 0.21                           | 0.26                    | 0.00                             |
| Ramachandran Plot            |                                |                         |                                  |
| Favored (%)                  | 96.51                          | 96.57                   | 95.40                            |
| Allowed (%)                  | 3.49                           | 3.43                    | 4.60                             |
| Disallowed (%)               | 0.00                           | 0.00                    | 0.00                             |
**Supplementary Table 2. Effects of mutations in the ligand-binding pocket of CCK₄R on CCK-8 binding affinities.**

Radiolabeled ligand ([¹²⁵I]CCK-8) binding assay was performed to evaluate the ligand-binding affinity of CCK₄R mutants. Binding data are represented mean pKi ± S.E.M. **P<0.01, versus wild-type (WT). N.D., not determined. FACS analyses were performed to evaluate the surface expression of the CCK₄R mutants. Expression data are shown as %WT. †P < 0.05, ††P < 0.01, †††P < 0.001, ††††P < 0.0001, versus WT. All data were analyzed by one-way ANOVA Dunnett multiple comparisons test. No adjustments were made for multiple comparisons.

| Mutant | pKi ± S.E.M. | n | P value | Expression % | n | P value |
|--------|--------------|---|---------|--------------|---|---------|
| WT     | 8.58 ± 0.12  | 4 | 1       | 100          | 3 | 1       |
| K105A  | 7.78 ± 0.22**| 3 | 0.0089  | 77.86 ± 6.24**| 3 | 0.0020  |
| F107A  | N.D.         | 3 | —       | 71.85 ± 6.84 | 3 | 0.0994  |
| T118A  | 8.73 ± 0.13  | 3 | 0.9921  | 78.06 ± 5.38' | 3 | 0.0153  |
| M121A  | 8.03 ± 0.15  | 3 | 0.1191  | 74.99 ± 5.48' | 3 | 0.0426  |
| V125A  | 8.68 ± 0.12  | 3 | 0.9993  | 46.48 ± 1.03***| 3 | <0.0001 |
| Y176A  | N.D.         | 3 | —       | 26.63 ± 2.43****| 3 | <0.0001 |
| F185A  | 7.98 ± 0.24  | 3 | 0.0742  | 77.98 ± 4.85 | 3 | 0.1029  |
| M195A  | 8.10 ± 0.26  | 3 | 0.2199  | 85.45 ± 4.52 | 3 | 0.5581  |
| C196A  | N.D.         | 3 | —       | 3.24 ± 0.06****| 3 | <0.0001 |
| R197A  | N.D.         | 3 | —       | 104.14 ± 5.14| 3 | 0.9993  |
| H210A  | 8.61 ± 0.12  | 3 | 0.9998  | 81.19 ± 4.32| 3 | 0.2350  |
| I329A  | N.D.         | 3 | —       | 74.22 ± 7.37' | 3 | 0.0334  |
| F330A  | 8.67 ± 0.09  | 3 | 0.9994  | 27.31 ± 2.74****| 3 | <0.0001 |
| A332G  | 8.43 ± 0.12  | 3 | 0.9921  | 32.50 ± 4.21****| 3 | <0.0001 |
| N333A  | N.D.         | 3 | —       | 63.96 ± 3.31***| 3 | 0.0009  |
| R336A  | N.D.         | 3 | —       | 82.96 ± 5.35 | 3 | 0.3489  |
| A343G  | N.D.         | 3 | —       | 50.61 ± 5.37****| 3 | <0.0001 |
| E344A  | N.D.         | 3 | —       | 88.00 ± 13.77| 3 | 0.7932  |
| L347A  | N.D.         | 3 | —       | 52.59 ± 1.43****| 3 | <0.0001 |
| S348A  | N.D.         | 3 | —       | 98.35 ± 8.18| 3 | 0.9997  |
| I352A  | N.D.         | 3 | —       | 80.35 ± 1.26| 3 | 0.1918  |
| Y360A  | 8.00 ± 0.09  | 3 | 0.0899  | 82.85 ± 6.85| 3 | 0.3411  |
Supplementary Table 3. Coupling activity of CCK\(_A\)R with different G proteins.

BRET assay was performed to evaluate the coupling activity of CCK\(_A\)R with different G proteins. Coupling activity data are represented as mean pEC\(_{50}\) ± S.E.M. Decreased fold of \(E_{\text{max}}\) compared to G\(_q\) was calculated. BRET experiments were performed in sextuplicate (n=6). Coupling activity data were analyzed by one-way ANOVA Dunnett multiple comparisons test. \(P\) values, versus Receptor + G\(_q\). Radiolabeled ligand binding assay was used to evaluate the allosteric effects of different G proteins on the binding affinity of CCK-8. The binding affinities are indicated as pKi ± S.E.M. Binding experiments were performed in triplicate (n=3). Binding data were analyzed by one-way ANOVA Dunnett multiple comparisons test. *\(P<0.05\), versus receptor.

| Group          | G protein-coupling activity of CCK\(_A\)R | Binding affinity of CCK-8 |
|----------------|------------------------------------------|---------------------------|
|                | pEC\(_{50}\) ± S.E.M. | n | \(P\) value | Decreased fold of \(E_{\text{max}}\) | pKi ± S.E.M. | n | \(P\) value |
| Receptor       | — | — | — | — | 7.92 ± 0.06 | 3 | 1 |
| Receptor + G\(_q\) | 8.42 ± 0.08 | 6 | 1 | 1 | 8.28 ± 0.08* | 3 | 0.0143 |
| Receptor + G\(_i\) | 7.32 ± 0.22 | 6 | 0.1230 | 6.60 | 7.87 ± 0.07 | 3 | 0.9148 |
| Receptor + G\(_s\) | 7.92 ± 0.65 | 6 | 0.5898 | 20.33 | 8.02 ± 0.06 | 3 | 0.6189 |
**Supplementary Table 4. Effect of I296G mutation of CCK₄R on G protein-coupling activity.**

BRET-based NanoBiT G-protein recruitment and NanoBiT G-protein dissociation assays were performed to evaluate Gq-, Gi-, and Gs-coupling activity, respectively. Data are represented as mean pEC₅₀ ± S.E.M. FACS analyses were performed to evaluate the surface expression of CCK₄R mutant. Radiolabeled ligand binding assay was used to evaluate the effects of the mutation on the binding affinity of CCK-8. The binding affinities are indicated as pKi ± S.E.M. All data were analyzed by two-tailed Student’s t-test by comparing I296G mutants with wild-type (WT) receptor. **P<0.01, versus WT. All experiments were performed in triplicate (n=3).

| Mutant | G protein-coupling activity | Cell Surface expression | Binding affinity |
|--------|-----------------------------|-------------------------|-----------------|
|        | pEC₅₀ ± S.E.M. | Expression % | pKi ± S.E.M. |
|        | Gq | Gi | Gs |               |               |
| WT     | 9.14 ± 0.04 | 6.81 ± 0.12 | 10.48 ± 0.10 | 100 | 8.58 ± 0.12 |
| I296G  | 8.38 ± 0.09** | 6.66 ± 0.08 | 10.46 ± 0.20 | 99.52 ± 3.10 | 8.63 ± 0.13 |
|        | 3 | 3 | 3 | 3 | 3 |
| n      | 3 | 3 | 3 | 3 | 3 |
| P value | 0.0015 | 0.3570 | 0.9330 | 0.8844 | 0.7915 |
Supplementary Fig. 1 | Cryo-EM workflows for structure determination of CCK₄R–G₉ protein complex. a, Size exclusion chromatography (SEC) profile and SDS-PAGE analysis of the CCK₈–CCK₄R–G₉–scFv16 protein complex sample. b, Representative cryo-EM micrograph (scale bar, 30 nm) and 2D classification averages (scale bar, 5 nm) of the CCK₈–CCK₄R–G₉–scFv16 complex. The data collection was performed once. The 2D averages display different secondary features in different views. c, Single-particle cryo-EM data processing flowcharts of the CCK₈–CCK₄R–G₉–scFv16 by Relion 3.1, including the Euler angle distribution of particles used in the final refinement and the fourier shell correlation (FSC) curves. The global resolution defined at the FSC=0.143 is 2.9 Å.
Supplementary Fig. 2 | Cryo-EM workflows for structure determination of CCKₐR–Gₛ protein complex.

a, Size exclusion chromatography (SEC) profile and SDS-PAGE analysis of the CCK-8–CCKₐR–Gₛ protein complex sample. b, Representative cryo-EM micrograph (scale bar, 30 nm) and 2D classification averages (scale bar, 5 nm) of the CCK-8–CCKₐR–Gₛ complex. The data collection was performed once. The 2D averages display different secondary features in different views. c, Single-particle cryo-EM data processing flowcharts of the CCK-8–CCKₐR–Gₛ by Relion 3.0, including the Euler angle distribution of particles used in the final refinement and the fourier shell correlation (FSC) curves. The global resolution defined at the FSC=0.143 is 3.1 Å.
Supplementary Fig. 3 | Cryo-EM workflows for structure determination of CCK₂R–Gₛ protein complex.

a, Size exclusion chromatography (SEC) profile and SDS-PAGE analysis of the CCK-8–CCK₂R–Gₛ–scFv16 protein complex sample. b, Representative cryo-EM micrograph (scale bar, 30 nm) and 2D classification averages (scale bar, 5 nm) of the CCK-8–CCK₂R–Gₛ–scFv16 complex. The data collection was performed once. The 2D averages display different secondary features in different views. c, Single-particle cryo-EM data processing flowcharts of the CCK-8–CCK₂R–Gₛ–scFv16 by Relion 3.0, including the Euler angle distribution of particles used in the final refinement and the fourier shell correlation (FSC) curves. The global resolution defined at the FSC=0.143 is 3.2 Å.
**Supplementary Fig. 4 | Receptor and Ga subunits used in the cryo-EM structure determination.**

**a,** A schematic illustration of the CCK<sub>4</sub>R construct used in cryo-EM studies. HA, hemagglutinin signal sequence; 2×MBP, double-MBP tag.  

**b,** Protein sequences of Ga<sub>q</sub>, Ga<sub>s</sub>, and Ga<sub>i1</sub> subunits. N-terminal sequence replaced in Ga<sub>s</sub> and Ga<sub>q</sub> is shown in blue. The two dominant-negative mutations are colored red and underlined. Stabilization mutations derived from the reported mini-Ga<sub>s</sub> are highlighted in cyan. AHD domain of the Ga<sub>s</sub> is replaced with the equivalent region of Ga<sub>i1</sub> and colored in gray.
Supplementary Fig. 5 | Local cryo-EM density maps of CCK₆R–G protein complexes. a, Cryo-EM density maps of TM1-TM7, ECL1-ECL3, ICL2, ICL3, CCK-8 peptide and α₅ helix of Gα₉ in the CCK-8–CCK₆R–G₉–scFv16 structure. b, Cryo-EM density maps of TM1-TM7, ECL1-ECL3, ICL2, CCK-8 peptide and α₅ helix of Gαₙ in the CCK-8–CCK₆R–Gₙ structure. c, Cryo-EM density maps of TM1-TM7, ECL1-ECL3, ICL2, CCK-8 peptide and α₅ helix of Gαᵢ in the CCK-8–CCK₆R–Gᵢ–scFv16 structure. d-f, The global density maps of the CCK-8–CCK₆R–G₉–scFv16 (d), CCK-8–CCK₆R–Gₙ (e), and CCK-8–CCK₆R–Gᵢ–scFv16 (f) colored by local resolution (Å). The density maps are shown at thresholds of 0.08, 0.055 and 0.05 for the CCK₆R–G₉, CCK₆R–Gₙ and CCK₆R–Gᵢ complex, respectively.
Supplementary Fig. 6 | Gating strategy of cell surface expression assay. Circle a gate E1 in the scatter map (red circle). The cells shown in the density map are all the cells in the gate E1 in the scatter map. Fluorescence signal intensity (FITC) is presented by density map. With the Blank sample as the reference value of background fluorescence signal (a), the “quadrant gate” divides the fluorescence signal density map into four quadrants. The third quadrant represents the negative cell community, while the fourth quadrant represents the positive cell community. The expression level of cell surface wild-type (WT) CCK₄R (b) can be calculated as follows: (M(Q2-4)-M(Q2-3))×(Q2-4% Parent). M, mean fluorescence intensity. The expression level of the CCK₄R mutant is calculated similarly to WT CCK₄R and then is normalized with the WT to calculate the relative expression value.